

**SYNTHESIS, DNA INTERACTIONS AND
CYTOTOXICITY OF NOVEL
CHLOROETHYLAMINOANTHRAQUINONES**

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For my parents
Hryhorij and Kateryna Paniwnyk

ABSTRACT

The design of DNA directed nitrogen mustards has led to the development of compounds with increased specificity for DNA and enhanced cytotoxicity compared to similar untargeted mustards. By linking an intercalating anthraquinone chromophore to a nitrogen mustard function it was anticipated that such compounds would also have the potential to irreversibly inhibit the topoisomerase II (topo II) enzyme. Fourteen anthraquinones containing various hydroxyethylaminoalkylamino side chains (hydroxyethylaminoanthraquinones) were synthesised, and ten were reacted further to give a series of mono, 5,8-dihydro or 5,8-dihydroxy nitrogen mustard anthraquinones (chloroethylaminoanthraquinones) substituted with alkylating side chains at the 1 or 1,4 position of the chromophore. The ability of hydroxyethylaminoanthraquinones to associate with DNA and to stabilize the helix to thermal denaturation was investigated. The increasing order of drug-helix stability was found to be 1-monosubstituted anthraquinone < 1,4-disubstituted-5,8-dihydroanthraquinone < 1,4-disubstituted-5,8-dihydroxyanthraquinone. In contrast, in the same DNA denaturation assay the chloroethylaminoanthraquinones showed apparent weak affinity for DNA, the exception being unsymmetrically substituted ZP281 which stabilised the helix by 10°C. Four chloroethylaminoanthraquinones ZP281, ZP282, ZP285 and ZP289 were selected for DNA covalent binding studies. These revealed that the compounds were excellent DNA inter-strand crosslinking agents that prevented strand separation at concentrations of 1nM. Furthermore, when compared to the non-targeted mustard mechlorethamine, the chloroethylaminoanthraquinones demonstrated an altered pattern of guanine alkylation which coincided with a preference for reaction at guanine sites with a pyrimidine 5' (5'PyG). This suggested that the chromophore influences the site of DNA alkylation and enhances the selectivity of the drug for DNA. To determine the cellular mechanism of the novel anthraquinones, a topo II inhibition study was performed in a yeast strain, *Saccharomyces cerevisiae* (*Sc. ce*), transfected with human topo II α or β , or yeast topo II. Of the seven compounds assayed three alkylating anthraquinones, ZP281, ZP285 and ZP289 inhibited yeast cell growth and may target both topo II α and β as a mechanism of cytotoxic action. ZP285 was the most potent compound requiring only 4.5 μ M to achieve 50% yeast cell kill. *In vitro* cytotoxicity assays were performed with ovarian carcinoma cell lines; A2780, CH1; cisplatin resistant cell lines A2780cisR, CH1cisR, SKOV-3; doxorubicin resistant cell line 2780AD; and mismatch repair chromosome transfer modified cell line B1. Seven compounds ZP257, ZP265, ZP275 (hydroxyethylaminoanthraquinones) and ZP281, ZP282, ZP285 and ZP289 (chloroethylaminoanthraquinones) were extremely potent in both wild type and resistant cells giving IC₅₀ values below 0.05 μ M. Furthermore, compound ZP281 displayed a relative lack of cross resistance in all resistant cell lines tested. The data in this thesis therefore indicates that ZP281 is a good candidate for further study as a potential covalent topoisomerase II inhibitor that may circumvent multi-drug resistance.

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- (1) *N,N*-Bis(2-hydroxyethyl)ethylenediamine
- (2) *N*-(2-Hydroxyethyl)-*N*-methylethylenediamine
- (3) *N*-(2-Hydroxyethyl)-*N*-methylpropanediamine
- (4) *N*-(2-Hydroxyethyl)-*N*-phenylethylenediamine

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- (6) 1-[*N*-[3-[*N*-(2-Hydroxyethyl)-*N*-methylamino]propyl]-amino]-9,10-anthracenedione (**ZP150**)
- (7) 1-[*N*-[3-[*N'*-*N''*-Bis(2-hydroxyethyl)amino]propyl]-amino]-9,10-anthracenedione (**ZP233**)

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- (9) 1,4-Bis-[*N*-[3-[*N',N'*-bis(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione (**ZP232**)
- (10) 1,4-Bis-[*N*-[3-[*N',N'*-bis(2-hydroxyethyl)amino]propyl]amino]-9,10-anthracenedione (**ZP240**)
- (11) 1,4-Bis-[*N*-[2-[*N'*-(2-hydroxyethyl)-*N'*-phenylamino]ethyl]amino]-9,10-anthracenedione (**ZP245**)

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- (13) 1,4-Bis[*N*-[3-[*N'*-(2-hydroxyethyl)-*N'*-methylamino]propyl]amino]-5,8-dihydroxy-9,10-anthracenedione (**ZP273**)
- (14) 1,4-Bis[*N*-[2-[*N',N'*-bis(2-hydroxyethyl)amino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione (**ZP274**)
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(18)	1-[<i>N</i> -[2-[<i>N',N'</i> -Bis(2-hydroxyethyl)amino]ethyl]amino]-4-[<i>N''</i> '-[2-[<i>N'''</i> ', <i>N'''</i> '-dimethylamino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione (ZP275)	
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(20)	1-[<i>N</i> -[3-[<i>N</i> -(2-Chloroethyl)- <i>N</i> -methylamino]propyl]amino]-9,10-anthracenedione hydrochloride (ZP288)	
(21)	1-[<i>N</i> -[3-[<i>N,N</i> -Bis(2-chloroethyl)amino]propyl]amino]-9,10-anthracenedione hydrochloride (ZP284)	
(22)	1,4-Bis[<i>N</i> -[2-[<i>N'</i> -(2-chloroethyl)- <i>N'</i> -methylamino]ethyl]amino]-9,10-anthracenedione dihydrochloride (ZP286)	
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(30)	1-[<i>N</i> -[2-[<i>N</i> -(2-Chloroethyl)- <i>N</i> -methylamino]ethyl]amino]-9,10-anthracenedione- <i>N</i> -oxide hydrochloride (ZP180)	

ABBREVIATIONS

ATP	Adenosine Triphosphate
γ^{32} P-ATP	Radiolabelled Adenosine Triphosphate
APS	Ammonium Persulphate
BAP	Bacterial Alkaline Phosphatase
dH ₂ O	Double distilled water
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
FAB-MS	Fast Atom Bombardment Mass Spectroscopy
I. R.	Infrared Spectroscopy
m. p.	Melting Point
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
N. M. R.	Nuclear Magnetic Resonance Spectroscopy
PCR	Polymerase Chain Reaction
<i>Sc. ce</i>	<i>Saccharomyces cerevisiae</i> yeast strain
<i>Taq</i> polymerase	<i>Thermus aquaticus</i> DNA polymerase
Topo	Topoisomerase enzyme
TEA	Tris/EDTA/Acetic Acid
TeoA	Triethanolamine
Tris	Tris(hydroxymethyl)aminomethane
TBE	Tris/Boric acid/EDTA
UV/ Vis	Ultraviolet and/or Visible Spectroscopy

Chapter 1 INTRODUCTION

1.1 CANCER AND ITS TREATMENT

Cancer is the second major cause of death in the UK. More than a quarter of a million people are diagnosed with it each year and based on national incidence rates it is estimated that at least 1 in 3 people will develop the disease during their lifetime. Survival rates vary enormously and depend on many factors including the type of cancer and at which stage it is treated. In the USA, the five year survival rate for women with early diagnosed breast cancer is over 80 per cent, whereas in the UK, the five year survival rate varies between 40-60% and is dependent on the patient's area of residence in addition to the initial stage of diagnosis and subsequent treatment. The probability of developing a particular type of cancer may in some cases be related to an individual's country of origin and their diet and lifestyle. This was evident when a survey revealed that the cumulative incidence rate (ages 0-74) of breast cancer varied more than six-fold over the world, and immigrants living in the USA that originated from countries with low risk breast cancer had an increased chance of developing the disease (Cancer Research Campaign Annual Report, 1997)

Cancer is defined as the uncontrolled proliferation of cells in which the physiological factors governing normal cell growth and tissue development become ineffective. Death in cancer patients commonly results from metastasis, which occurs when cancer cells invade surrounding tissues and the lymphatic system. Cancer cells can thus be carried to take residence as secondary tumours distant from the primary tumour. The new tumour can continue to grow by stimulating the development of its own vasculature (angiogenesis) (reviewed by Vaupel *et al.*, 1989; Brown *et al.*, 1998) and, depending on the site of growth, death will eventually occur.

To treat cancer with maximum effect a combination of surgery, if appropriate, with radiotherapy and/or chemotherapy is used. Chemotherapy is usually administered over a period of weeks or months and generally comprises of a number of cytotoxic drugs with different modes of action and activity in tumours. Because chemotherapeutic

drugs are also toxic towards normal dividing cells, it is important to compare both the long and short term toxicities of each drug individually in order to balance the clinical benefit to the patient against potential side-effects. Often chemotherapy results in severe systemic toxicities which can be dose limiting and can produce side effects including nausea, vomiting, myelosuppression, reproductive sterility and alopecia. Tumours can also acquire resistance to drug therapy. This commonly occurs if repeated small doses of drug are given over a long period of time. Therefore, in some cases where it is determined that the patient is able to withstand such treatment, it can be beneficial to administer an initial high dose of the drug in order to maximise cell kill.

Although there are problems associated with chemotherapy, certain tumours such as Hodgkins disease, acute lymphoblastic leukaemia in children, and teratoma of the testis in adults are curable. However, many cancers are still non-responsive to available treatments and can be alleviated only temporarily. Thus there is an overwhelming need to develop new drugs that can be targeted more effectively towards tumour cells and do not display the toxic side effects associated with most existing cancer treatments.

1. 2 THE TOPOISOMERASE II ENZYME AND ITS ROLE IN DRUG ACTION

1. 2. 1. CELLULAR ROLE AND STRUCTURE

DNA exists as a highly complex structure packaged tightly within chromosomes as loops, knots, supercoils and catenanes. In order for the cell to function correctly, the regulation of DNA topology is of critical importance. Eukaryotic topoisomerases are enzymes that achieve this by altering the conformational state of DNA. At least two forms of topoisomerase exist, type I and type II, referred to as topo I and topo II respectively. These enzymes are able to relax supercoiled DNA by introducing transient single (topo I) or double (topo II) strand breaks into the helix of either different DNA molecules or to different regions of the same DNA molecule, and then by passing a second intact helix through the strand break (Liu *et al* ., 1980; Gellert, 1981; Liu, 1983;

Sternglanz *et al.*, 1989). Topo II is known to be involved with DNA transcription and replication (Brill *et al.*, 1987) and its close association with nuclear processes implies that it has a number of important cellular functions. For example, it has been identified as the major non-histone protein which links the basis of the radial chromatin loops to the central axis of mitotic chromosome fiber, and it is involved with chromosome condensation and separation of DNA daughter chromatids at the end of replication (Di Nardo *et al.*, 1984; Earnshaw *et al.*, 1985; Uemura *et al.*, 1986; Adachi *et al.*, 1991). More recently, it is thought to function as part of the DNA repair process (Larson *et al.*, 1998). The enzyme topo I is predominantly located in the nucleolar region in proliferating cells and is associated with genes and regions which are undergoing active transcription by RNA polymerase I or II (Muller *et al.*, 1985; Capranico *et al.*, 1992). Topo I is continuously expressed throughout the cell cycle and therefore may be an effective target in slow growing tumours that are currently resistant to chemotherapy (Heck *et al.*, 1988). Recently a newly identified topo III has been isolated as a single copy gene located at chromosome 17p11.2-12. The function of topo III has yet been fully described, although it was shown to successfully substitute for topo I in yeast cells lacking endogenous topo I (Hanai *et al.*, 1996).

Eukaryotic DNA topo II exists as two isoforms, α and β , with a molecular weight of 170kD and 180kD, respectively. Each isoform is coded on separate chromosomes and is regulated differently during the cell cycle by enzyme specific phosphorylation (Tsai-Pflugfelder *et al.*, 1988, Jenkins *et al.*, 1992, Tan *et al.*, 1992; Corbett *et al.*, 1992 and 1993). The α isoenzyme is under strict cell cycle control. It is minimally expressed in G1 and in quiescent cells, but goes on to increase exponentially through the S phase, eventually reaching maximal expression in late G2 phase (Chow *et al.*, 1987). In contrast, topo II β is expressed in low constant concentration throughout all phases of the cell cycle (Woessner *et al.*, 1991). Because of these differences in cell cycle distribution it is generally considered that each isoenzyme is targeted separately by topo II inhibitors. Topo II α particularly is suggested to be the major target of inhibitors that arrest cells at G2/M phases (Chen *et al.*, 1995; Qui *et al.*, 1996b). Furthermore, because both are expressed differentially in proliferating and non-proliferating tissue, and are located at different sites within the cell nucleus, for example, topo II β has been

reported to be nucleolar, although this has more recently been disputed (Meyer *et al.*, 1997), growing evidence suggests that the isoenzymes also perform separate functions within the cell (Capranico *et al.*, 1992a; Zini *et al.*, 1994).

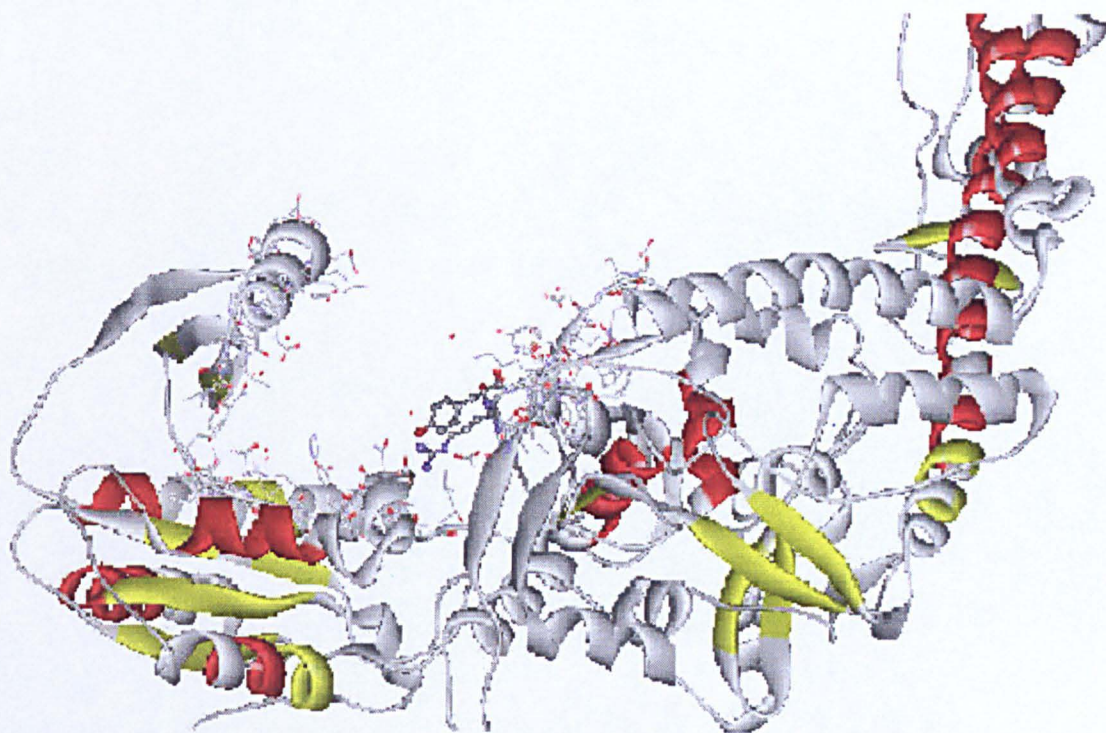


Fig. 1. 1 A restricted length of topo II, amino acids 410-1202. The active tyrosine is represented as a ball and stick configuration.

Both of the human topo II α and β cDNAs have been cloned and their respective proteins can be divided into three conserved consensus sequences. Namely, an N-terminal ATP-ase binding domain, a central breakage reunion domain (which contains the reactive tyrosine, amino acid 804) and a C-terminal domain containing a leucine zipper motif. The isoenzymes show high nucleotide sequence homology (approximately 72%) until the C-terminal domain where only approximately 35% sequence homology exists (Austin *et al.*, 1993). Crystal structure analysis of yeast topo II shows that it consists of two crescent shaped monomers joined together to form a heart shaped dimer which surrounds a large hydrophobic central hole. At the DNA cleavage site the hydrophobic centre of the enzyme is accessible by the unpaired nucleotides from each DNA strand (Fig. 1. 1) (Roca *et al.*, 1992; Berger *et al.*, 1996; Roca *et al.*, 1996, Capranico *et al.*, 1997).

Topo II cleavage sites are not randomly distributed along DNA, but are restricted to regions encompassing loosely defined nucleotide sequences of approximately 15 base pairs (Spitzner *et al.*, 1988). Preferential cleavage has been identified at alternating purine-pyrimidine bases, independent of DNA conformation (Spitzner *et al.*, 1989; Choi *et al.*, 1995). Furthermore, the initial recognition of DNA by topo II is thought not to be restricted to sites on both DNA strands as the two subunits may act cooperatively to effect cleavage. Hence, only one molecule of drug is required to be associated with the DNA-enzyme complex to create a double strand break in DNA (Bigioni *et al.*, 1994).

1. 2. 2. THE CATALYTIC CYCLE OF TOPOISOMERASE II

The catalytic activity of topo II is manifest through a DNA double strand passage two gate mechanism for which an ATP modulated molecular clamp has been proposed (Roca *et al.*, 1992). Initially each enzyme monomer binds non-covalently to each DNA strand producing a species known as the 'non-cleavable complex' and establishing a DNA cleavage/ religation equilibrium. A trans-esterification reaction between an enzyme tyrosine group with each strand of DNA produces a pair of 5' end covalently linked DNA-protein phosphodiester bonds and a DNA break (G-segment) four base pairs apart. This is known as the 'cleavable complex' (Liu *et al.*, 1983). The binding of ATP and the presence of a divalent metal ion co-factor creates a conformational change in the enzyme. This induces the protein to capture a second DNA strand (T-segment) which enters through one gate (N-gate) in the enzyme, passes through the 'G-segment' and finally out of a second gate in the enzyme (C-gate) which lies opposite to the site of entrance (Roca *et al.*, 1996). A second trans-esterification to release the original bound DNA results in a post-strand passage cleavage/ religation equilibrium and is followed by hydrolysis of ATP to regenerate the original conformational state of the enzyme and to trigger enzyme turnover. This mechanism has been defined by its ready conversion to a protein-linked DNA strand break on exposure to alkali or detergent, both strong protein denaturants (Liu *et al.*, 1983).

1. 2. 3. TOPOISOMERASE II AS A POTENTIAL DRUG TARGET

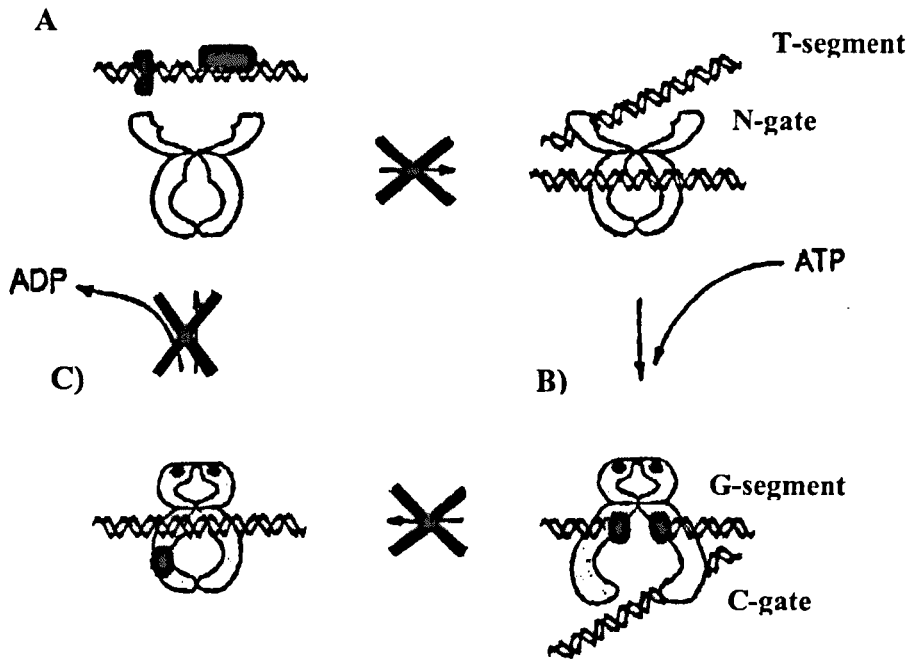


Fig. 1. 2 The effect of different types of inhibitor on specific stages of the catalytic cycle of topoisomerase II: A) 'DNA binding inhibitors'- prevent the enzyme from binding to DNA. B) 'Topo II poisons'- stabilize the cleavable complex and hinder the resealing of strand breaks C) 'Enzyme binding inhibitors'- block protein conformational transitions. The black oblong/circle represent the sites of various drug action. From Capranico *et al.*,1997.

Topo II is the cellular target for a wide variety of anti-neoplastic agents. Intercalating drugs such as the anthracyclines and anthraquinones (see section 1. 3. 1 and 1. 3. 2), and also the non-intercalating epipodophyllotoxins e.g etoposide (VP-16) and teniposide (VM-26), primarily act by stabilizing the cleavable complex and shifting the rate of equilibrium towards its formation (see Fig. 1. 2B)) (Nelson *et al.*, 1984; Robinson *et al.*,1990). Both these type of drug are commonly referred to as 'topo II poisons' as they convert the enzyme into a DNA damaging agent by increasing the probability of DNA double strand break production which is potentially a lethal event.

Although double strand breaks are known to initiate cell death, the exact mechanism of inhibitor cytotoxicity is yet to be established. Cleavable complex stabilising drugs are generally most cytotoxic to cells in the S phase of the cell cycle i.e

during DNA synthesis. However, they cause the greatest number of strand breaks in the G2 /M phase, where topo II expression is maximal (Chow *et al.*, 1987). Furthermore, once an inhibitor is removed from its site of action the DNA strand breaks are reversed (Tewey *et al.*, 1984; Hsiang *et al.*, 1989). These observations signify that the drug-topo II-DNA ternary complex itself is not cytotoxic and that other cellular processes such as ongoing DNA replication may be required to develop the cytotoxic effects of these agents. Early studies with the topoI inhibitor camptothecin demonstrated that lethal lesions may be generated if transient DNA breaks are converted to permanent lesions when replication forks attempt to traverse the protein-bound blockages in the DNA (Hsiang *et al.*, 1988). Direct evidence of this as a mechanism of cytotoxicity arising from topo II inhibition was established in Human Leukaemia CEM cells by (Catapano *et al.*, 1997) who showed that replication fork (RF) arrest inhibited chain elongation, and in the *c-myc* oncogene occurred specifically at the site of topo II mediated cleavage (Qui *et al.*, 1996a).

More recently, topo II inhibitor related DNA-strand breaks have been associated with the onset of apoptosis. The addition of etoposide to immature rat thymocytes, caused a concentration dependent fragmentation and laddering of DNA, a clear indication of the self destruction of the cell and programmed cell death (apoptosis) (Sun *et al.*, 1994). Whether or not this was a consequence of replication fork arrest is unknown.

The cytotoxicity of an inhibitor can in most cases be correlated with the frequency of DNA strand breaks that it produces (Long *et al.*, 1984). When this relationship is compared between individual inhibitors at a known concentration of drug, it becomes evident that each DNA break does not result in the same cytotoxic potency. For example, the anthracycline doxorubicin creates fewer lesions yet is more cytotoxic than etoposide (Paoletti *et al.*, 1993). This may be explained by the rate of lesion repair following the removal of drug from the target site (Ross *et al.*, 1982) or the duration of exposure of tumour cells to the drug induced cleavable complex. (Fox *et al.*, 1990; Smith *et al.*, 1990).

Further studies using DNA sequence specific analysis identified that drugs of the same class produce a consistent, characteristic cleavage in the presence of topo II. Some

sites were found to be identical to those created by the enzyme alone, whilst others were entirely new. At the most intense sites the base located either 5' or 3' of the DNA break

Table 1. 0 Base specificity of anti-cancer drug interaction with topoisomerase II

Drug	Preferred base location*		References
	- 1	+1	
Doxorubicin		A	Capranico <i>et al.</i> , 1990a
<i>m</i> -AMSA		A	Pommier <i>et al.</i> , 1991a
VM 26, VP 16	C(T)		Pommier <i>et al.</i> , 1991a
Mitoxantrone	C(T)		Capranico <i>et al.</i> , 1993

* Denotes base position in relation to DNA break (-1 = 5', +1 = 3')

was unique to the inhibitor (Table 1. 0). From this evidence it was postulated that effective drug interactions are likely to depend on the local DNA sequence (Paoletti *et al.*, 1993). This principle was extended to relate the exact location of the complexes in the genome with drug potency. The high affinity of *m*-AMSA for the *c-myc* oncogene promoter region was shown to coincide with enhanced topo II-DNA cleavage (Pommier *et al.*, 1992). Similarly, in a study of anthracyclines, the most active compounds induced prominent cleavage in only a small region of SV40 DNA (Capranico *et al.*, 1990b).

A lack of drug sensitivity has in some cases been linked to low levels of topo II enzyme within certain tumour types. For example, cancers such as chronic lymphocytic leukaemia and colon carcinoma are poorly responsive to these agents (Potmesil *et al.*, 1988). Attempts have therefore been made to enhance inhibitor cytotoxicity by stimulating the topo II content of tumour cells. Recent studies have demonstrated that the addition of topotecan, a topoI inhibitor, to athymic mice bearing SW480 human colon carcinoma xenografts increased topo II α levels and sensitivity to the topo II inhibitor etoposide (Utsugi *et al.*, 1997). Others have found that the addition of Tumour Necrosis Factor together with either the topoI inhibitor camptothecin or topo II inhibitors such as doxorubicin produced a dose-dependent synergistic cytotoxicity

against murine L929 fibrosarcoma cells. This combination was identified with a rapid and transient increase in the respective topoisomerase which resulted in an increased number of strand breaks and cytotoxicity (Utsugi *et al.*, 1990).

1.3 DEVELOPMENT OF CHEMOTHERAPEUTIC DRUGS

1.3.1 ANTHRACYCLINES

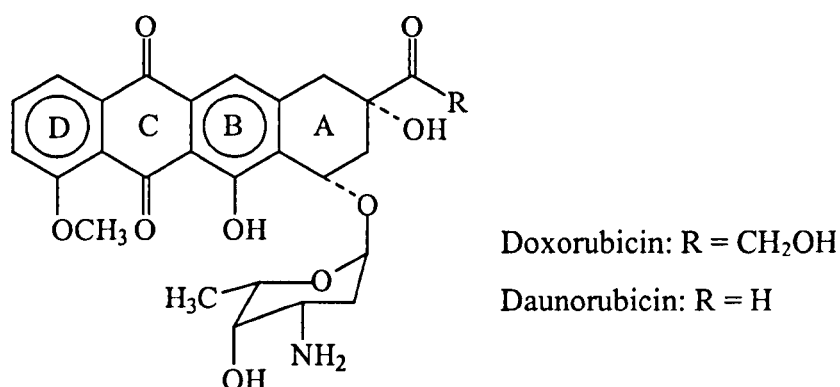


Fig. 1.3 The anthracyclines doxorubicin and daunorubicin.

An important advance in tumour therapy was the discovery and isolation of daunorubicin, the first antibiotic of its class to show activity against acute lymphoblastic leukaemia in man (Jacquillat *et al.*, 1966). Doxorubicin, (see Fig. 1.3) an analogue of daunorubicin, exhibited the same kind of inhibitory effect on tumour growth, but was generally more potent and has since become one of the most established and widely used drugs in the clinic today. It is effective in the treatment of breast cancer and leukaemia, and has a broad spectrum of activity against a number of solid tumours (Arcamone *et al.*, 1985). As with most anti-cancer drugs a disadvantage in the use of anthracyclines is their toxicity towards non-tumour cells. Prolonged use of doxorubicin can lead to the acute dose limiting effect of myelosuppression, and to cumulative dose related cardiotoxicity which is manifest through drug related free radical generation (Olson *et al.*, 1990). This limits the total dose of drug that can be given to a patient in their lifetime and hinders the utility of these agents in single or in combination therapy.

Whilst early studies also correlated the cytotoxicity of the anthracyclines with the induction of free radical mediated tissue damage *in vitro* and *in vivo* (Lown *et al.*, 1982; Kappus, 1986), further investigation suggested the contrary. Research by Fisher *et al.*, (1989 and 1992) demonstrated that the drug mitoxantrone which also includes a quinone chromophore did not undergo redox cycling and despite this, was more cytotoxic than doxorubicin to MCF7 human breast cancer cells. By analogy, it was proposed that free radical production may only play a small role in the cytotoxicity of doxorubicin.

The chemical structure of both doxorubicin and daunomycin comprises of an aglycone ring coupled to a daunosamine sugar (see Fig 1. 3) which allows the drug to bind to DNA via the reversible process of intercalation (see Fig 1. 4). The mechanism

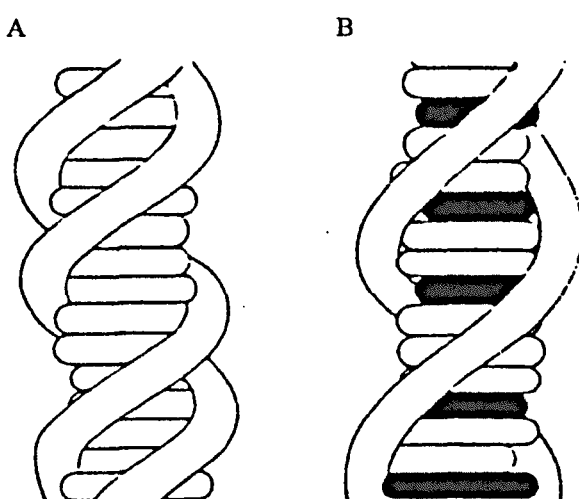


Fig 1. 4 Comparison of a) B-DNA with b) B-DNA following intercalation. The drug is represented by the shaded areas of the helix. A model proposed by Lerman, 1961.

of intercalation was first proposed by Lerman in (1961) who hypothesised that the base pairs of DNA were co-planar but could be separated by an additional 3.4 angstroms to accommodate a planar acridine chromophore. The close contact between π -orbitals of the drug molecule and the base pairs stabilise the complex by stacking interactions, which are essentially van der Waals forces, hydrophobic interactions and charge transfer forces. As the drug becomes intercalated in between the base pairs, the DNA begins to

forces. As the drug becomes intercalated in between the base pairs, the DNA begins to locally unwind and stiffen, bend and elongate which leads to a disruption of the regular helix structure (Reinert *et al.*, 1983). At high drug concentration the consequence of this is directly to affect DNA-protein interactions (see section 1. 3. 2. 3).

At the site of intercalation the long axis of the anthracycline chromophore is seen to lie almost perpendicular with that of the DNA base pairs. Rings B and C overlap with adjacent base pairs, whilst the A ring passes through the intercalation site into the minor groove. The daunosamine sugar also lies in the minor groove (Wang *et al.*, 1987). Studies have indicated that anthracyclines have an intercalation sequence preference at GCA or GCT triplet base pairs and have strong binding affinity with DNA (Pullman, 1991). Despite this, intercalative binding has proved to be insufficient for antitumour activity, instead, the cytotoxicity of anthracyclines is manifest through the stabilisation of the DNA/Topo II cleavable complex (Tewey *et al.*, 1984a; Capranico *et al.*, 1989) (see section 1.2). This was supported by the observation that a doxorubicin derivative with a weaker DNA binding chromophore (idarubicin) was more effective at inducing topo II related cleavage, implicating the chromophore and not the sugar moiety in topo II inhibition (Capranico *et al.*, 1989 and 1990b).

1. 3. 2. ANTHRAQUINONES IN THE TREATMENT OF CANCER

1. 3. 2. 1 MITOXANTRONE

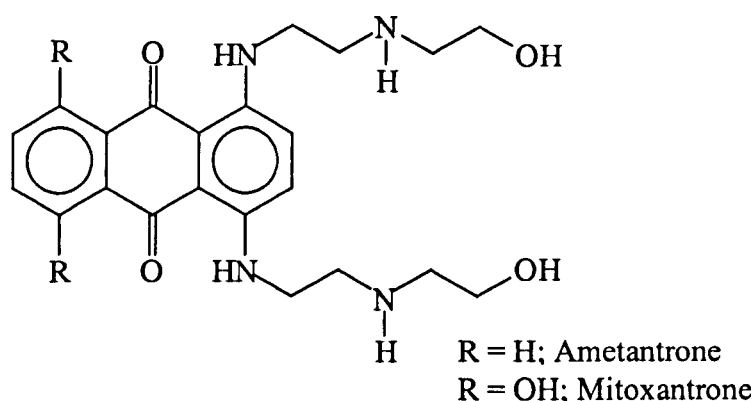


Fig. 1. 5 The anthraquinones Mitoxantrone and Ametantrone

In an attempt to identify compounds which lacked the toxicities associated with doxorubicin but at the same time retained its spectrum of activity, research led to the synthesis of two lead compounds: Mitoxantrone, 1,4-Bis{*N*-[2-[*N'*-(2-hydroxyethyl)-amino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione and its 5,8-dihydro derivative ametantrone (see Fig. 1.5) (Zee-Cheng *et al.*, 1978; Johnson *et al.*, 1979; Murdock *et al.*, 1979). Both compounds comprise of a tricyclic aromatic quinone chromophore disubstituted on the 1 and 4 positions with an ethanolamine containing side chain. The anthraquinones retain only one feature from the anthracycline structure, that is, a planar aromatic chromophore as it is essential for drug intercalation into DNA (Kapuscinski *et al.*, 1981, Lown *et al.*, 1985). Since its isolation in 1979 mitoxantrone has proved to be effective in the clinic and is currently used in the treatment of breast cancer, lymphoma and acute leukaemia (Koeller *et al.*, 1988). In addition to having a broad spectrum of activity it also has reduced cardiotoxicity in comparison to doxorubicin (Herman *et al.*, 1997).

The first studies of mitoxantrone showed that it inhibited DNA synthesis and RNA processing (Johnson *et al.*, 1979; Kapuscinski *et al.*, 1981; Foye *et al.*, 1982; Cheng *et al.*, 1983). Its cytotoxicity was found to coincide with the aggregation and compaction of chromatin (Kapuscinski *et al.*, 1986) and the creation of protein associated DNA single and double strand breaks which correspond with the drug stabilisation of both DNA-topo I and II enzyme complexes (see section 1. 3. 2. 3) (Tewey *et al.*, 1984b; Crespi *et al.*, 1986).

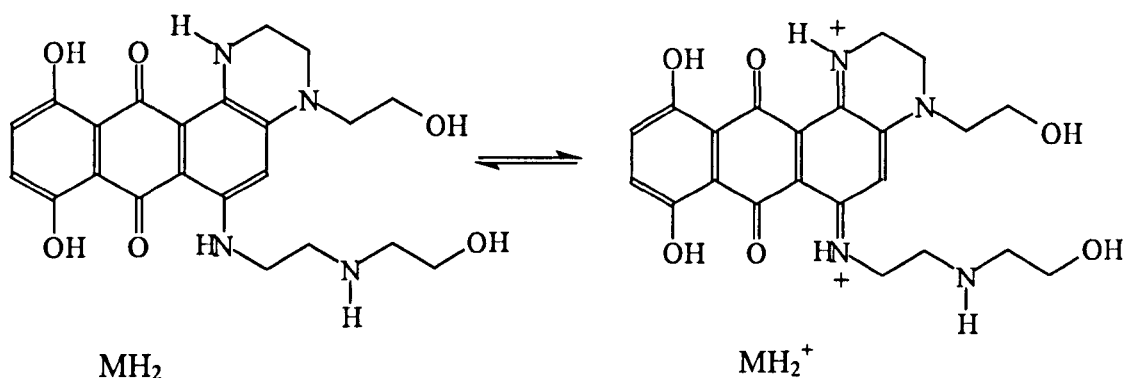


Fig. 1. 6 MH_2 and its rearrangement to the diimino metabolite, MH_2^{2+} .

The detection of mitoxantrone metabolites following the analysis of human urine samples established that it is capable of undergoing oxidative activation to a number of highly electrophilic species. These include the product of intramolecular rearrangement, hexahydronaphtho{2,3-f}quinoxaline-7,12-dione (MH₂) (see Fig. 1. 6) (Rezka *et al.*, 1986; Chiccarelli *et al.*, 1986). MH₂ can bind DNA covalently and is thought to contribute to the non-protein associated DNA single strand breaks created following drug treatment (Reska *et al.*, 1989; Panoussis *et al.*, 1995).

1. 3. 2. 2. STRUCTURE AND BINDING

Mitoxantrone binds to DNA biphasically, by intercalation and by the weaker electrostatic interaction of its basic amino side chains with the anionic phosphate groups of the DNA helix (Kapuczinski *et al.*, 1981; Collier *et al.*, 1988; Pohle *et al.*, 1990). With the use of computer modelling three intercalative binding modes for mitoxantrone have been proposed. In the first mode the drug lies parallel to the long axis of the DNA base pairs with one sidechain in each groove, 'straddling' the intercalation site. The second model shows the chromophore lying perpendicular to the DNA base pairs with both side chains lying in the major groove (Fig. 1.7b) (Islam *et al.*, 1985; Collier *et al.*, 1988; Tanious *et al.*, 1992). Recently a third mode was proposed (Mazerski *et al.*, 1998). This arrangement requires both sidechains of the drug to thread through to the minor groove with the chromophore lying perpendicular to the long axis of the DNA. Once intercalation has occurred, it is suggested that the small width of the minor groove slows the rate of drug disengagement from DNA and promotes longer retention times which can be directly related to mitoxantrone's high potency. Despite this evidence, the general agreement is that the perpendicular mode is the most favourable binding configuration for the 1,4 di-substituted anthraquinones and that it is the 5,8 hydroxyl groups of mitoxantrone that slow its disengagement from the minor groove (Denny *et al.*, 1990).

To further attempt to understand the high affinity of mitoxantrone for DNA evidence of its sequence selectivity and base pair preference have been determined. The computer modelling of specific DNA nucleotide sequences with mitoxantrone have

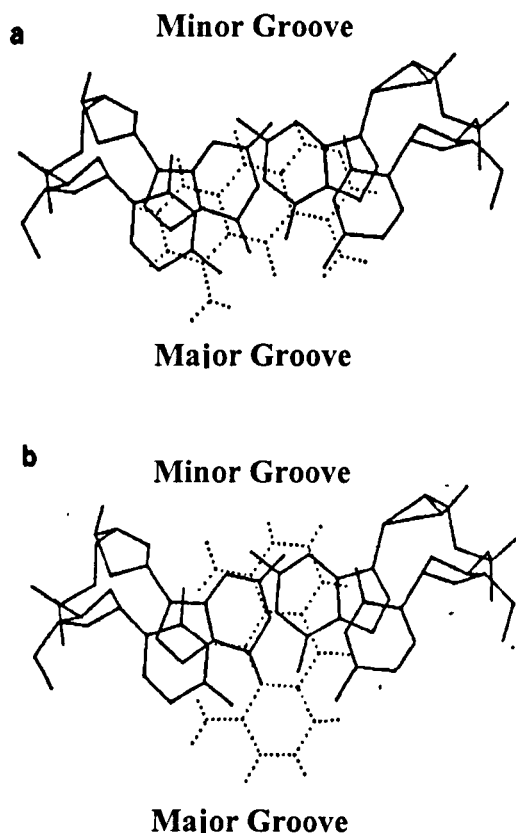


Fig 1. 7 Shows chromophore a) parallel and b) perpendicular intercalation into DNA. Figure obtained from Islam *et al.*, 1985.

reported preferential intercalation at GC sites (Fox *et al.*, 1986; Chen *et al.*, 1986). Whilst further work with DNase footprinting studies show preferential binding at 5'(A/T)CG and 5' (A/T)CA base pairs (Panoussis *et al.*, 1994). In a comparative DNase footprinting study, mitoxantrone but not ametantrone was found to select for the 5'(A/T)CG triplet sequence suggesting that in this case the chromophore hydroxyl groups may influence the site of intercalation (Bailly *et al.*, 1996). Although mitoxantrone has demonstrated some preference for GC base pairs, it has been suggested that a slow disengagement from GC sites may in fact be due to their reduced rate of opening compared to AT sites (Malhotra *et al.*, 1980).

1. 3. 2. 3. TOPO II INHIBITION AND CELLULAR MECHANISMS OF ACTION

The most important mechanism of mitoxantrone cytotoxicity is the inhibition of topo II. At low concentrations mitoxantrone is a potent topo II inhibitor (see section 1.

2. 3). Its cytotoxicity corresponds with G2 cell cycle arrest and the long term trapping of the topo II cleavable complex (Roberts *et al.*, 1989; Fox *et al.*, 1990; Smith *et al.*, 1990). Conversely, at high drug concentration ($> 1\mu\text{M}$) mitoxantrone loses its cell cycle specificity and a dose dependent decrease in DNA strand breaks is observed through intercalation mediated distortion of the helix. At this point mitoxantrone becomes an enzyme catalytic inhibitor. Topo II is prevented from binding DNA because the potential sites of cleavable complex formation are saturated with drug (see Fig. 1. 2A) (Smith *et al.*, 1990). Confocal spectral imaging of K562 erythroleukaemia cells to localise the cellular effects of mitoxantrone established that it binds in monomeric and aggregate form to both nuclear and cytoplasmic structures and its persistent drug action is governed by the dissociation of the cytoplasmic aggregates to the nucleus (Feofanov *et al.*, 1997, Smith *et al.*, 1990 and 1992). Furthermore, as a direct effect of high mitoxantrone concentration the initiation of apoptosis and cell death has been observed, indicating an enhanced mode of cytotoxicity, probably through drug saturation of the cellular target (Feofanov *et al.*, 1997; Bhalla *et al.*, 1993).

1. 3. 2. 4. MITOXANTRONE ANALOGUES

A wide range of anthraquinone analogues have been synthesised and evaluated including compounds that have undergone bis-substitution onto the 2 and 6, and 1 and 8

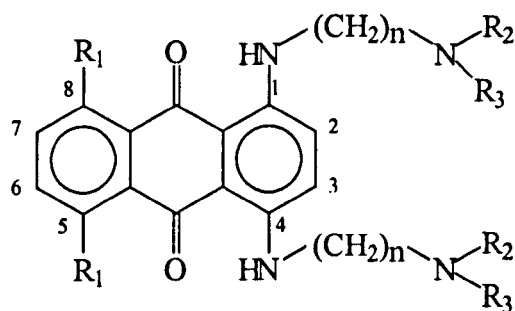


Fig 1. 8 Modified positions on the anthraquinone chromophore and side chains

positions of the chromophore (Murdock *et al.*, 1979; Johnson *et al.*, 1979; Collier *et al.*, 1988, Agbanjie *et al.*, 1992). These studies revealed that the ideal requirement for potent anti-tumour activity is the substitution of two side chains onto the 1 and 4 positions and also hydroxyl groups onto the 5 and 8 positions ($R_1 = \text{OH}$) of the

chromophore. Additionally, each side chain should retain its cationic nature and contain an ethylene (C2) alkyl spacer ($n = 2$) between its two amino groups (see Fig. 1. 8) (Denny *et al.*, 1990). Although this is exemplified by mitoxantrone which remains amongst the most potent within its class of drug, comparative studies into the effects of varying the sidechain length ($n \geq 2$) and substituents (R_2 and R_3) of a number of anthraquinone derivatives has not revealed a clear correlation between structure and biological activity. For example, the sequential addition of a methylene group to increase side chain length coincided with a decline in tumour activity, despite the increased lipophilicity of the compounds (Johnson *et al.*, 1979; Cheng *et al.*, 1983). Increasing the number of hydroxyethyl groups on the sidechain from two to four ($R_2 = R_3 = -CH_2CH_2OH$) weakens the affinity of the drug for DNA and correlates with a decline in cytotoxicity through increased drug-water solubility (Murdock *et al.*, 1979). In contrast, the synthesis of 1, 4- bis{*N*-[2-*N,N'*-(dimethylamino)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione (AQ4) which contains two tertiary amino sidechain substituted with four *N*-methyl groups ($R_2 = R_3 = CH_3$) produced a highly lipophilic compound which binds to DNA more efficiently than mitoxantrone, evident by its higher thermal melting temperature and DNA association constant (Murdock *et al.*, 1979; Patterson, 1993). Hence, although drug structures can give a good indication of anti-tumour activity they cannot always be relied upon to give an optimum determination of drug potency.

1. 3. 2. 5. ASYMMETRIC ANTHRAQUINONES

In a study to evaluate the influence of sidechains on DNA binding, a number of unsymmetrically substituted 5, 8 dihydro anthraquinones were prepared. Krapcho *et al.*, (1986) found that although some showed excellent activity *in vitro* they were mostly inactive *in vivo*. Subsequent work resulted in the synthesis of 1-[*N*-[2-(*N,N'*-dimethylamino)ethyl]amino]-4-[*N*-[2-(*N''''*hydroxyethylamino)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione also known as (AQ6) (see Fig.1. 9). This proved to be the most active analogue and was found to be more cytotoxic than mitoxantrone and

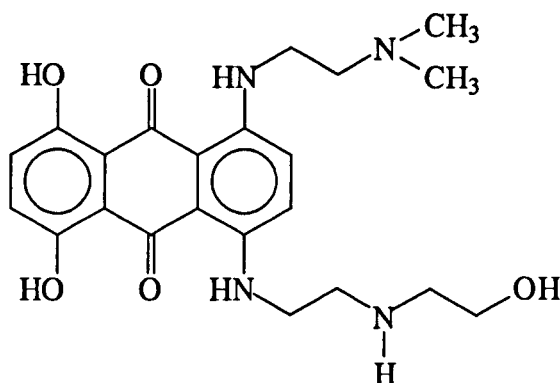


Fig 1.9 AQ6

doxorubicin in both sensitive and resistant human colon carcinoma sublines (Krapcho *et al.*, 1990). More recently, the cytotoxicity of AQ6 has been identified with S phase cell cycle kill and linked to the inhibition of the enzyme topo II (Smith *et al.*, 1997). The rationale behind the unexpectedly high activity of AQ6 was thought to be because the optimal hydrophobic-lipophilic balance had been achieved with this drug structure. It was also concluded that identical sidechains are not essential for anti-tumour action as the level of activity depends on the nature of the second arm (Stefanska *et al.*, 1990).

1. 4 NITROGEN MUSTARDS AND ANTI-TUMOUR DRUG DEVELOPMENT

1. 4. 1 THE EARLY ORIGINS OF CANCER THERAPY

The origin of cancer chemotherapy can be traced back to the First World War and to the use of mustard gas (sulphur mustard) in the battlefields. At that time the ability of mustard gas to induce specific action on the bone marrow was attributed to its production of hydrochloric acid in intracellular regions presumed to be hypersensitive to acidity. Once its promising anti-tumoural effect was realised, the difficulty in containing sulfur mustard was overcome by the development of a compound with similar chemical and biological properties, nitrogen mustard. This compound could be stored as a stable hydrochloride salt and could easily be neutralised to produce an active nitrogen mustard

free base. As a result, the nitrogen mustards became the first drugs to be used in the trials of systemic therapy. In 1946 Gilman and Philips were first to describe the alkylation chemistry of the sulphur and nitrogen mustards and to summarise their effect on biological systems. Since then, the simplicity of the nitrogen mustard structure and its potential for chemical variation has led to the synthesis of hundreds of derivatives including cyclophosphamide (Brock *et al.*, 1963), melphalan (Bergel *et al.*, 1954), and chlorambucil (Everett *et al.*, 1953), three drugs that continue to play a major role in current anti-cancer chemotherapy.

1. 4. 2. CLASSIFICATION AND MODE OF ACTION

Nitrogen mustards are a member of the alkylating, anti-neoplastic agents which have an ability to react with biologically important macromolecules through the formation of highly electrophilic intermediates. Alkylation can occur by two nucleophilic substitution mechanisms: S_N1 or S_N2 and is dependent on the nature of the substituents attached to the alkylating moiety (reviewed by Niculescu-Duvaz *et al.*, 1991). In the case of mechlorethamine

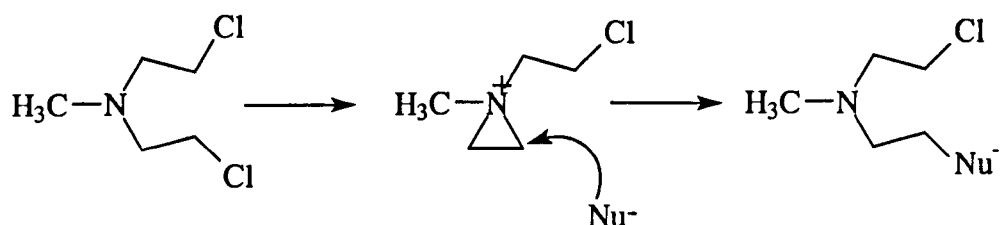


Fig 1. 10 S_N2 reaction of mechlorethamine via aziridinium ion formation.

(also known as nitrogen mustard) the S_N2 mechanism predominates and reaction occurs via the formation of a positively charged aziridinium ion intermediate (see Fig 1. 10). Because the bases of DNA are susceptible to alkylation at physiological pH it was believed that the biological effects of alkylating agents result from such reactivities (Brookes *et al.*, 1960). Specific alkylation sites on DNA were identified by Pullman *et al.*, (1981) by comparing the molecular electrostatic (MEP) of each DNA base. The

most negative site on DNA and hence the most reactive site was found to be at the guanine N⁷ position with less reactive sites at the O⁶ of guanine and N¹ and N³ adenine.

The detection of crosslink formation emerged from the isolation of nitrogen mustard linked guanine dimers (Lawley *et al.*, 1965). Since then, covalent binding to DNA has been defined by inter-strand crosslinks (alkylation at two bases each on opposite DNA strands), intra-strand crosslinks (alkylation of two bases on the same DNA strand) and mono adducts (a single base alkylation). The proportion of each adduct formed can vary between agents. For mechlorethamine the majority of the adducts appear to be monoalkylations at guanine N⁷, with inter-strand crosslinks between GN⁷-GN⁷ accounting for 4-7% of total adduct formation (Kohn *et al.*, 1966). Adducts at adenine N³ have also been observed following reaction with mechlorethamine and melphalan, although to a lesser extent (Osborne *et al.*, 1993 and 1995). Studies to identify base pair sites of nitrogen mustard inter-strand crosslink formation have demonstrated that complementary 5'GN*C (N*=any base) sequences are favoured with 5'GC sites being less prevalent (Rink *et al.*, 1993; Rink *et al.*, 1995b). A mechlorethamine 5'GN*C inter-strand crosslink has been shown by computer modelling to bend the DNA duplex by 12.4-16.8° per lesion thus altering the structure of DNA and in turn potentially modulating its affinity for DNA binding proteins (Rink *et al.*, 1995a).

Generally, the formation of inter-strand crosslinks is considered to be the most effective way to create lethal lesions as intra-strand crosslinks are more likely to undergo error free repair (see section 1. 5. 1. 4) (Lawley *et al.*, 1996). Whilst monoadducts are described as being a determinant of mutagenic damage rather than cytotoxic effect (Povirk *et al.*, 1994). The importance of bifunctional crosslinking agents is their potential to create inter-strand di-adducts with DNA and despite the fact that these crosslinks are less abundant they are generally considered to be the major cytotoxic lesion as they may lead to DNA double strand breaks and cell death .

1. 4. 3. DNA SEQUENCE SPECIFICITY OF NITROGEN MUSTARDS

Nitrogen mustards are known to have a characteristic nucleotide alkylation preference for guanine bases, more particularly, Pullman *et al.*, (1981) found that a

guanine situated within a run of three guanines would be expected to be more reactive than the other two guanines. This was confirmed by Kohn *et al.*, (1987) who established a relationship between molecular electrostatic potential (MEP) and the reaction intensity of a number of nitrogen mustards. For example, mustards such as mechlorethamine which contain a high positive charge, react intensely at guanine N⁷ positions. In comparison, chlorambucil which also contains a negative charge within its structure rendering it electrically neutral reacts to a lesser extent but still at the same sites. Not all alkylating agents follow this principle, in some cases the substituent attached to the alkylating moiety can introduce distinct sequence preferences for reaction. Uracil mustard was seen to prefer 5'PyGC sites over runs of guanines and mustards attached to intercalating chromophores were more selective towards alkylation at particular guanines (Mattes *et al.*, 1986a; Kohn *et al.*, 1987; Ponti *et al.*, 1991).

Agents that modify DNA covalently can block, at specific sites, the progress of both DNA and RNA polymerases (Ponti *et al.*, 1991; Gray *et al.*, 1991). The processing of these induced blockages by either DNA repair (see section 1. 5. 1. 4) or the activation of cell death can determine the outcome of cytotoxicity. In murine leukaemia cells exposed to cisplatin (a platinating agent which can inter-strand crosslink DNA), long-standing arrest in G2 and subsequent cell death coincided with the appearance of DNA double strand breaks (Sorensen *et al.*, 1988). Others have postulated that due to their sequence selectivity, nitrogen mustards may specifically target GC rich regions of the genome. These include the 5' flank control regions of a number of protooncogenes (Hartley *et al.*, 1988). Analysis of alkylated sites within the the 5' flank of the c-Ha-ras oncogene showed that these were preferred sites of damage by a number of guanine N⁷ specific alkylating agents (Mattes *et al.*, 1988). Furthermore, mechlorethamine was able to inhibit the binding of transcription factors such as NFkB and SP1 to their GC rich consensus sequences but not those that bind to AT rich sequences (D'Incalci *et al.*, 1993). More recently, studies have indicated that actively transcribing regions/strands of DNA are modified preferentially by DNA binding agents because of their increased accessibility, however this may be dependent on the nature of the lesions and their location in the genome (Larminat *et al.*, 1993; Wasserman *et al.*, 1994a; and 1994b).

Nitrogen mustards are used in the treatment of many cancers such as Hodgkins disease, breast cancer and ovarian cancer (reviewed by Black *et al.*, 1990 and 1990a). Their short half-life and high reactivity results in a rapid removal from the site of administration and the non-specific targeting of rapidly growing tissues. Overall, only a low concentration of drug reaches its target and is often found secreted within the liver and kidneys. Consequently, nitrogen mustards are particularly subject to dose related toxicity (Connors *et al.*, 1986). As many have been classified as potential mutagens and potential human carcinogens, it is important to establish a balance between therapeutic efficacy (cell killing) and unwanted side-effects (mutation induction).

1. 4. 4. DNA TARGETED ALKYLATING AGENTS

1. 4. 4. 1. ACRIDINE LINKED MUSTARDS

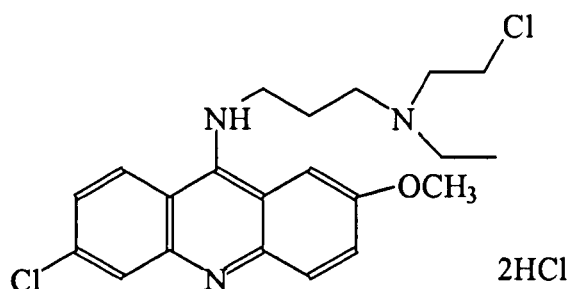


Fig 1. 11 2-methoxy-6-chloro-9-[N-3-(N'-ethyl-(N''-2-chloroethyl)-amino)propyl]aminoacridine dihydrochloride

Bifunctional alkylating agents are highly reactive compounds with low specificity for DNA. One way to overcome this lack of selectivity is to attach a DNA affinic chromophore to the alkylating moiety. Although initial studies proved successful, over the years relatively few groups have focused on the development of DNA targeted nitrogen mustards.

In 1959, Peck *et al* synthesised a number of monofunctional alkylating agents attached to various heterocyclic chromophores. One particular mustard, 2-methoxy-6-chloro-9-[N-3-(N-ethyl-N'-2-chloroethylamino)propyl]aminoacridinedihydrochloride (see Fig. 1. 11) showed good activity by prolonging the survival rate of mice bearing ascites tumours. Furthermore, it also exhibited mutagenic capability in *Drosophila*. As

the corresponding bis mustards also displayed highly improved cytotoxicity the heterocyclic nucleus and 2-chloroethyl groups were identified as being of critical importance. A further fifty mono and bifunctional derivatives with varying sidechain length and acridine chromophore were synthesised and tested. From this research it was concluded that a DNA affinic chromophore could influence the activity of a nitrogen mustard group by directing it towards and intercalating into DNA (Peck *et al.*, 1961, Preston *et al.*, 1964). Further studies by the same group confirmed that a tricyclic or tetracyclic aromatic chromophore was required for high antitumour activity and as a result a mechanism was proposed suggesting that initial intercalation was followed by the alkylation of DNA bases by the chloroethyl group (see Fig 1.12) (Creech *et al.*, 1972).

More recently, Ferguson *et al.*, (1989) synthesised a number of mono and bifunctional aniline mustards attached to a 9-aminoacridine chromophore by a linker chain of varying length. By also varying the substituent attached immediately to the aniline ring, the reactivity of the aromatic mustard with DNA could be altered. When the cytotoxicity of these compounds was determined in DNA repair proficient and deficient strains the compounds were found to be considerably more potent in the repair deficient strains (>10 fold) suggesting that DNA crosslinking was the major mechanism of action.

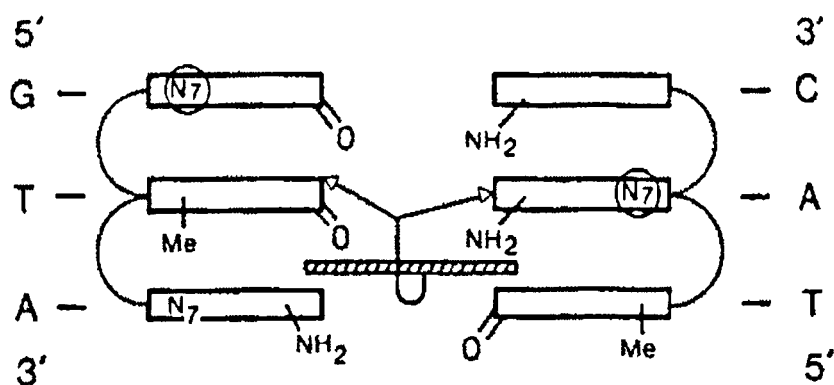


Fig 1. 12 Quinacrine intercalated in between DNA base pairs. The circled N7 bases indicate potential sites for alkylation and inter-strand crosslink formation. The shaded rectangle represents the acridine chromophore. From Prakash *et al.*, (1990).

In a study comparing the cytotoxicities of 9-aminoacridine mustards against untargeted mustards in murine leukaemia P388 and AA8 and UV4 CHO derived cell lines up to a 100 fold increase in potency was observed for the targeted mustards. These compounds also showed good *in vivo* activity in mice bearing the p388 leukaemia cell line (Ferguson *et al.*, 1989; Valu *et al.*, 1990; Gourdie *et al.*, 1990).

Other groups have linked aliphatic nitrogen mustards to acridine chromophores. The linker chain of 9-[N-[n-[N-(2-chloroethyl)-N-methylamino]alkyl]amino]-2-methoxy-acridine dihydrochloride was varied in length (alkyl (CH₂)_n; (n) = 2-6). When the cytotoxicity of the targeted mustards was compared to the parent compound mechlorethamine in human colon carcinoma HT-29 cells a 10-100 fold increase in potency was observed for the targeted mustards (Kohn *et al.*, 1994).

Attempts have also been made to improve the low crosslink to mono adduct ratio by synthesizing agents that could potentially alkylate both grooves. A series of 9-aminoacridine-4-carboxamide derivatives substituted with an aniline mono-mustard at the 4- and 9-position of the chromophore were instead found to preferentially form monoadducts at guanine-N7 (Gourdie *et al.*, 1991). Other examples of targeted bifunctional crosslinking agents are the platinating acridine derivatives (Palmer *et al.*, 1990; Mikata *et al.*, 1998) and aniline mustard amsacrine derivatives (Fan *et al.*, 1997).

1. 4. 4. 2 MORPHOLINO DOXORUBICIN ANALOGUES AS ALKYLATING AGENTS

One of the more promising series of drugs to emerge recently are the morpholino doxorubicin derivatives. These compounds were originally synthesised by Acton *et al.*, (1984) in an attempt to overcome the toxicities and drug resistance associated with doxorubicin. They vary from doxorubicin in structure by a substituted morpholino ring at the 3' position (R) of the sugar moiety (see Fig. 1.13). *In vitro* analysis of the morpholino derivatives revealed that they are mostly non-cardiotoxic at effective anti-tumour doses (Sikic *et al.*, 1985) and display a lack of cross-resistance in doxorubicin resistant cell lines exhibiting MDR1 (drug resistance through the overexpression of the membrane glycoprotein, Pgp) and at-MDR (drug resistance through an altered

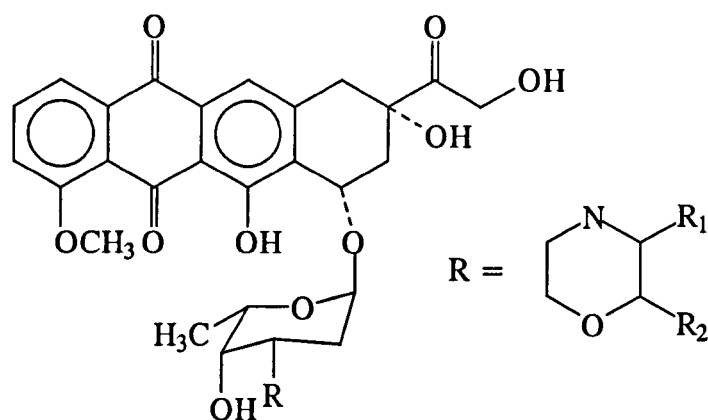


Fig. 1. 13 Morpholino doxorubicin derivatives MRA-CN and MMDX

topoisomerase II enzyme) (see section 1. 5. 1. 1. and 1. 5.1. 2.), and in cisplatin resistant cell lines (Streeter *et al.*, 1986, Bielack *et al.*, 1995; van der Graaf *et al.*, 1995). MRA-CN in particular is extremely cytotoxic, demonstrating between 100-1500 fold potency over doxorubicin (Johnston *et al.*, 1983; Bielack *et al.*, 1995). It has been postulated that the substituted hydrophobic morpholino ring increases the lipophilic character of these compounds and facilitates their transport through the cell membrane. MDR1 is avoided by effectively altering the drug affinity for P-glycoprotein (Pgp), a drug efflux pump, and so a greater concentration of drug is accumulated and retained in both sensitive and resistant cells (Acton *et al.*, 1984; Johnston *et al.*, 1987; Coley *et al.*, 1993). Despite this, in doxorubicin resistant cell line P388/ADR, the addition of verapamil (a Pgp inhibitor) restored not only the cytotoxicity of doxorubicin but also enhanced the cellular uptake of 4-Methoxymorpholino doxorubicin (MMDX) and 3'-deamino-3'-(3-cyano-4-morpholinyl) doxorubicin (MRA-CN) although to a considerably lesser extent. These observations suggest that MDR1 is not completely circumvented by these agents (Streeter *et al.*, 1986). MRA-CN is a DNA alkylating agent with the potential to crosslink DNA. Unlike MMDX does not require metabolic activation (Scudder *et al.*, 1988; Jesson *et al.*, 1989). X-ray diffraction analysis demonstrated that the aglycone of

MRA-CN is intercalated between CG base steps with its morpholino ring lying in the minor groove (Gao *et al.*, 1995). Site specific alkylation studies have revealed that

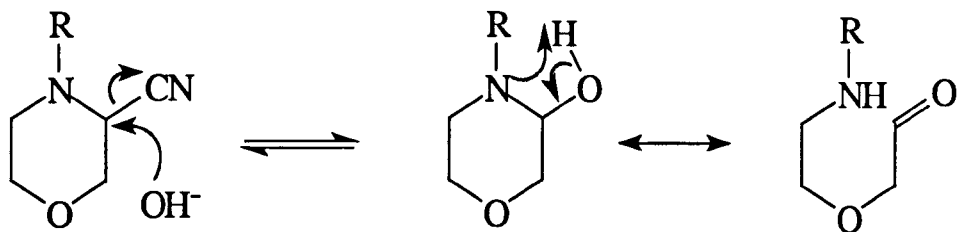


Fig. 1. 14 Proposed ring opening reaction of cyano morpholino to alkylate DNA via Schiff's base formation

MRA-CN has a preference for runs of guanines at 5'GG sequences which are the sites of intra-strand crosslink formation, with less selectivity for 5'GC base pairs (Cullinane *et al.*, 1991). More recently a mechanism by which MRA-CN alkylates DNA has been proposed whereby the drug undergoes structural rearrangement with the loss of the cyano group and the opening of the morpholino ring to alkylate DNA via an iminium ion intermediate (see Fig. 1.14).

Whilst doxorubicin is known to inhibit topo II, studies indicate that MRA-CN has a cytotoxicity profile which mirrors that of camptothecin a topoI inhibitor, particularly at high concentration (Wasserman *et al.*, 1990). This may in part explain the ability of MRA-CN to avoid at-MDR.(Bielack *et al.*, 1995)

1. 5 MULTI DRUG RESISTANCE MECHANISMS ASSOCIATED WITH ANTHRAQUINONE AND NITROGEN MUSTARD ANTI-CANCER AGENTS

1. 5. 1. THE MULTI DRUG RESISTANCE (MDR) PHENOTYPE

Multi drug resistance (MDR) is a major hindrance to the successful treatment of neoplastic disease. It is the process whereby the exposure to one drug induces cross-resistance to a variety of agents to which the cell has not yet been exposed. MDR can be intrinsic, conferring a cell's natural resistance to cytotoxic agents, or it can be acquired

through drug exposure. Generally, it is intrinsically resistant cells are the most difficult to treat as these often develop into a subpopulation of cells that maintain the resistance phenotype.

In vitro studies have shown that MDR cells may differ from their drug sensitive counterparts by a number of factors: 1) Decreased accumulation of cytotoxic drugs and net drug binding; 2) changes in the expression of particular cellular proteins and 3) enhanced repair mechanism. These differences can occur individually or, as in many cases in combination (multifactorial resistance). It is therefore essential to understand how a tumour cell develops strategies to evade the cytotoxic effects of anticancer drugs in order to overcome the problem of MDR.

1. 5. 1. 1 MDR1 AND MRP MEDIATED RESISTANCE

To date there are two major mechanisms of resistance, MDR1 and MRP, that are associated with the overexpression of membrane glycoproteins. Resistance has been shown to vary from *in vitro* to *in vivo* clinical settings, and has been linked in some cases to the overexpression or amplification of the *MDR1* gene which encodes for P-glycoprotein (Pgp) (Holzmayer *et al.*, 1992) and/or of the *MRP1* gene which encodes the multidrug resistance-associated protein (MRP) (Kavallaris, 1997). Pgp and MRP are both energy dependent efflux pumps which act by binding and exporting drug from the cell in order to protect it from cytotoxic damage. The detrimental effect of this is to prevent sufficient quantities of chemotherapeutic drug from reaching its target.

Despite only a 15% homology between their structures they are members of the same ATP-binding cassette superfamily of membrane proteins (Cole *et al.*, 1992) and are capable of conferring resistance to a similar though not identical, multiple range of chemotherapeutic drugs. These include natural products such as the epipodophyllotoxins, the anthracyclines and the vinca alkaloids (Grant *et al.*, 1994). Their apparent identical function does not make them mutually exclusive as studies with the small cell lung cancer cell line, H69, have indicated that *MRP* and *MDR1* may co-exist in the same cell type (Brock *et al.*, 1995).

Pgp has been isolated as an ATP-driven, trans membrane transporter protein with a molecular weight of 170kD (Endicott *et al.*, 1989). *In vitro* levels of *MDR1* gene

expression have shown correlation with the degree of resistance within a cell type and transfection of *MDR1* into drug sensitive cells is sufficient to confer the MDR1 phenotype (Veda *et al.*, 1987). However, where no initial overexpression of Pgp has been observed it has been suggested that exposure of cells to cytotoxic drugs can induce the MDR1 phenotype (Baas *et al.*, 1990).

The *MRP1* gene was cloned from a non-Pgp mediated multidrug resistant small cell lung cancer cell line and was found to code for an 190kDa membrane protein (Cole *et al.*, 1992). It is expressed in a wide variety of normal and tumour tissues at both RNA and protein level (Stride *et al.*, 1996). Low MRP mRNA expression has been detected in the brain, liver and small intestine, with moderate to high levels in lung, skeletal muscle, testis and ovary (Kruh *et al.*, 1995). Despite this, the role of MRP in the failure of chemotherapy is poorly defined and so evidence of its true influence on MDR has yet to be established.

However, recent studies of resistance have identified a possible role for MRP as the coordinator of an ATP-dependent glutathione *S*-conjugate carrier (GS-X pump), which is responsible for transporting glutathione and glucuronide conjugates out of the cell. Evidence for this mechanism was acquired by comparing levels of expression of *MRP1* with the ATP-dependent transport of glutathione conjugated compounds (Jedlitschky *et al.*, 1996; Muller *et al.*, 1994) and further substantiated by the finding that MRP expressing resistant cells accumulated more drug following treatment with buthionine sulfoximine (BSO), an agent that inhibits glutathione synthesis (Versantvoort *et al.*, 1995). Others have found that the suppression of *MRP1* expression results in hypersensitivity to natural cytotoxins but surprisingly not to alkylating agents which are specific substrates for intracellular glutathione conjugation (Lorico *et al.*, 1997). In support of this, Morrow *et al.*, (1998) demonstrated that MRP alone failed to confer resistance to the nitrogen mustard chlorambucil, however, the combined expression of *MRP1* with glutathione-*S*-transferase isoenzyme A1-1 (α -GST) acted in synergy to protect cells from the same bifunctional crosslinking agent. Hence, it has been postulated that MRP may participate in both drug detoxification and glutathione metabolism.

1. 5. 1. 2 ALTERED TOPOISOMERASE II

Overexpression of drug efflux pumps results in the removal of drugs from the cell before they reach their cellular target. Many topo II inhibitors are substrates for these efflux pumps and consequently resistance develops through decreased interaction with the enzyme topo II. However, alterations in the topo II enzyme itself may also induce resistance and in some cases the altered topoisomerase (at-MDR) mechanism has been shown to exist without *MDR1* and *MRP1* expression. In the absence of efflux pumps the drug is effectively excluded from the nucleus and is found sequestered in cytoplasmic structures away from its site of action (Zwelling *et al.*, 1990; Whithoff *et al.*, 1996; Consoli *et al.*, 1997).

Cell lines conferring the at-MDR phenotype have been shown to form a reduced number of cleavable complexes which in turn leads to a lower frequency of strand breaks and a decrease in cytotoxicity (Harker *et al.*, 1991; Fry *et al.*, 1991). Others have demonstrated that differences in sensitivity between drug classes in resistant cells may be dependent on the ratio of topo II α to β in the cell, and to the isoenzyme preference of a compound. For example, cells made resistant to topo II inhibitor VM26 correlated with altered topo II α levels (Mirski *et al.*, 1993). Whilst low sensitivity to mitoxantrone has identified with a decrease in topo II β , other groups have disputed this preference (Harker *et al.*, 1991; Hammonds *et al.*, 1998).

The topo II status of a cell is also an important determinant of resistance. Slow growing tumours, which are notoriously difficult to treat, generally reside in the G0/G1 phase of the cell cycle where topo II α levels are mostly undetectable (Potmeisil *et al.*, 1988; Woessner *et al.*, 1991). As a result, several stress conditions such as hypoxia and glucose starvation have been linked to poor drug response through low topo II levels (Harris *et al.*, 1992).

at-MDR can occur as a function of reduced catalytic activity, reduced levels of the enzyme, and mutations and deletions (Deffie *et al.*, 1989; Yu *et al.*, 1997). In HL-60/MX2 cells, resistance to mitoxantrone was defined by a decrease in topo II catalytic activity, undetectable amounts of topo II β and the isolation of a novel 160kDa topo II α protein located in the cytoplasm (Harker *et al.*, 1991). Further studies identified that the novel protein was associated with a truncated topo II α mRNA causing a disruption of

the carboxy terminal of the enzyme and altering its cellular localisation to the cytoplasm (Harker *et al.*, 1995a and 1995b, Mirski *et al.*, 1995). Hence, drug cleavable complexes were formed only with the reduced levels of topo II α in the nucleus. In support of this, it has been postulated that cytoplasmic topo II may also act as a drug 'sump' delaying the drug from entering the nucleus (Whithoff *et al.*, 1996). A number of mutations associated with drug resistance have been identified within the topo II α gene. These include alterations at the ATP binding domain which may impair the binding of ATP to the enzyme, and those near the active tyrosine which may prevent drug and/or topo II from binding to DNA. It is unknown whether these mutations are located within the drug interaction domain of topo II as many mutant enzymes display different and contradictory profiles of drug resistance (Osheroff *et al.*, 1994). Furthermore, whilst *in vitro* experiments demonstrate the involvement of atMDR in drug resistance, there is little evidence to support gene mutation as a mechanism resistance in clinical specimens (Nitiss *et al.*, 1996; Hsiung *et al.*, 1996).

Studies of topo II cellular levels and sensitivity to alkylating agents have generally shown an inverse correlation (Campling *et al.*, 1997). For example, the increased resistance of Burkitt lymphoma cells to mechlorethamine coincided with reduced levels of DNA inter-strand crosslink formation and an approximate 4-fold increase in topo II activity compared to the sensitive cell line (Tan *et al.*, 1987). Whilst in cisplatin resistant L1210 murine leukaemia cells an increase in sensitivity towards topo II inhibitor amsacrine correlated with a 3-fold increase in topo II activity. Following the sequential addition of the two drugs an additive effect was seen in the sensitive cell line and a supra additive effect in the resistant cells (Barret *et al.*, 1994). These results imply a causal relationship between topo II expression and resistance towards alkylating agents and is suggested to be linked to topo II α involvement with the damage recognition and repair of DNA (Larsen *et al.*, 1998).

1. 5. 1. 3 DETOXIFICATION BY GLUTATHIONE AND ITS ROLE IN RESISTANCE

The glutathione-S-transferases (GST's) are a family of cytosolic proteins that can catalyse the conjugation of xenobiotics and their electrophilic metabolites with the

endogenous tripeptide glutathione (GSH) (reviewed by Tew *et al.*, 1994). For some nitrogen mustards the conjugation reaction with glutathione can also take place non-enzymatically, the rate of the reaction increasing with pH (Bolton *et al.*, 1993). As indicated in section 1. 5. 1. 1, GSH affects the efficacy and action of a variety of antineoplastic agents mainly through nucleophilic thioether formation or oxidation-reduction reactions and thus plays a role in the detoxification and repair of cellular injury. Mammalian GST isoenzymes have been grouped into three major classes α , μ and π on the basis of structural and functional characteristics (Armstrong *et al.*, 1991). Early detection of resistance to mechlorethamine demonstrated that reduced inter-strand crosslinking in resistant Chinese Hamster Ovary (CHO) cells correlated with a three fold overexpression of GST activity in the absence of deficient repair or reduced drug accumulation (Robson *et al.*, 1987). Elevated levels of GSH and GST were also found to contribute to the decrease in sensitivity to mechlorethamine in 9L rat brain tumour cells (Evans *et al.*, 1987). More recently resistance to certain alkylating agents has been linked to the overexpression of one or more GST isoenzymes. Increased amounts of α -GST have been observed in cell lines resistant to mechlorethamine (Lewis *et al.*, 1988; Clapper *et al.*, 1989). In contrast, the transfection of α -GST into MCF7 human breast cancer cells had no effect on sensitivity to chlorambucil, melphalan or cisplatin, inferring that this particular isoenzyme is not a contributing factor in the resistance to these drugs (Leyland-Jones *et al.*, 1991). These inconsistencies suggest that a loss of sensitivity may perhaps depend on the cell type and the individual drug used, and has led to the true role of GSH in the detoxification of alkylating agents to be questioned. When no GSH-melphalan (L-PAM) conjugates were detected in rat or human perfused liver samples it was postulated that GSH may play a role in the repair of drug induced DNA damage rather than act as a detoxifying agent (Vahrmeijer *et al.*, 1996, Britten *et al.*, 1991).

Glutathione is also involved in the detoxification and resistance to the anthracyclines and the anthraquinones. It has been implicated in the detoxification of mitoxantrone metabolites through conjugation with products from metabolic oxidation of its sidechains and anthraquinone chromophore (Mewes *et al.*, 1993). MDR cell lines selected for doxorubicin resistance have displayed elevated or altered GSH levels (Deffie *et al.*, 1988; Dajani *et al.*, 1995) and the overexpression of π -GST isoenzyme

(Lee *et al.*, 1989). The cellular increase in GST is not always accompanied by overexpression of Pgp (Lee *et al.*, 1989; Cole *et al.*, 1990) and because as it is not uniform within MDR cell lines its contribution to anthracycline resistance is thought to be secondary to that of Pgp MDR (Beck *et al.*, 1990).

1. 5. 1. 4 DNA REPAIR

The exact mechanism by which bifunctional DNA adducts are detrimental to the cell is unknown. Recent studies have indicated that it is likely that abortive attempts to replicate and/or repair damaged DNA leads to the formation of double-strand breaks or single strand gaps in the DNA causing chromosome aberrations and breakage during mitosis (Chaney *et al.*, 1996). Conversely, another response to DNA damage is for the cell to trigger a significant increase in proficient adduct repair, the outcome being the creation of fewer cytotoxic lesions and resistance to chemotherapeutic agents. Lesions identified with nitrogen mustard DNA damage are mostly single base alkylations which produce monoadducts, and to a lesser extent, inter- and intra-strand DNA crosslinks (see section 1. 4. 1. 1). The response to such lesions is to activate one or more of the repair mechanisms available to the cell (see Fig 1.15).

A) In direct repair, the cytotoxic drug methylation of the O⁶ position of a guanine (or O⁴ position of a thymine) base is directly repaired. That is, the cytotoxic lesion is removed from the base by transferring the alkyl group to a specific cysteine residue of the repair protein methylguanine-DNA methyltransferase (MGMT). The damaged base is restored without any further modification (Mittra *et al.*, 1993).

B) Base-Excision Repair (BER) and its role in repairing damage caused by alkylating agents involves the enzyme methylpurine-DNA glycosylase (MPG) (Sancar, 1994). This enzyme is active against alkylated purines by removing the damaged base from the DNA. Apurinic/apyrimidinic (AP) lyase followed by AP endonuclease then act to release the deoxyribose sugar. The resulting gap is filled by DNA polymerase β (and to a lesser degree polymerase δ and ϵ) and closed by DNA ligase. BER, has been identified by the enhanced removal of inter-strand crosslinks and elevated levels of MPG, and has been implicated in the resistance of lymphocytes from CLL (chronic lymphocytic leukaemia) patients receiving nitrogen mustard treatment (Bramson *et al.*,

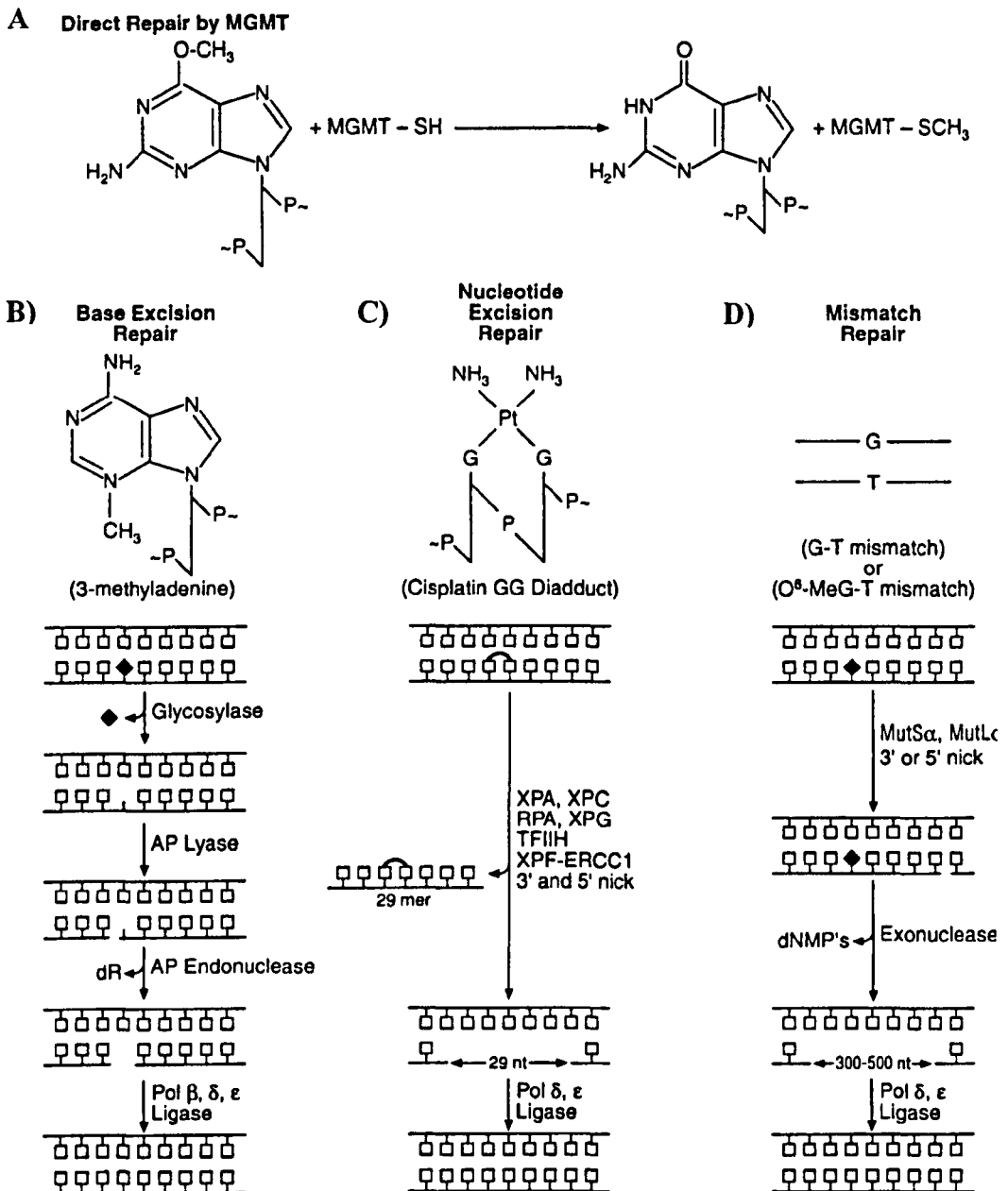


Fig 1. 15

DNA repair mechanisms a) Direct Repair b) Base Excision Repair c) Nucleotide Excision Repair d) Mismatch Repair. RPA = Replication repair factor; TFIIH = transcription/repair factor; XPA, XPC, XPG, XPF-ERCC1 = products of genes corresponding to xeroderma pigmentosum (XP) or excision repair cross-complementing (ERCC) groups; Pol = DNA Polymerase; dNMP's = deoxynucleoside monophosphate. From Chaney *et al.*, (1996).

1995; Geleziunas *et al.*,1991).

C) Nucleotide Excision Repair (NER) is dependent on ATP and the combination of six repair factors to remove lesions from DNA. The DNA damage created is incised on both sides of the lesion and excised as a 29-nucleotide long oligomer. The resulting gap is filled with DNA polymerase δ or ϵ and closed with DNA ligase (Sancar *et al.*, 1994; Mu *et al.*, 1995). NER is suggested to be the most likely mechanism of repair of bifunctional DNA damage because the creation of inter and intra-strand crosslinks involves more than one base (Chaney *et al.*, 1996). The overexpression of the repair factor ERCC1 (excision repair cross complementing group 1) was found to be associated with resistance to mechlorethamine in CHO cells and also to have a negative effect on sensitivity to cisplatin in human ovarian carcinoma cells (Bramson *et al.*.,1993; Li *et al.*, 1998 and 1999).

D) Mismatch repair (MMR) plays a vital role in the postreplicative correction of DNA polymerase errors that have escaped proofreading during DNA replication, and in the control of the fidelity of genetic recombination (Modrich *et al.*, 1996). It is different from the other repair systems as the lesions removed are undamaged nucleotides. Instead, the abnormality arises because the base is mispaired. For example, the deamination of methyl-cytosine produces a G.T mispair which will be further converted to a G.A mispair if a thymine is not correctly substituted for the cytosine prior to DNA replication (Griffin *et al.*, 1994). MMR involves the excision of a long tract of DNA followed by synthesis of a repair patch of up to 1000 nucleotides. Exonucleolytic degradation of DNA is thought to be initiated from a nick in the strand generated from Okazaki fragments during the synthesis of the defective DNA strand (Modrich *et al.*, 1996). To date five genes that encode mismatch repair proteins have been identified. In normal human cells mispaired bases are recognised by two complexes: hMutS α (containing the hMSH2 subunit) and hMutS β (human MutS homologue). These can activate the hMutL α (the human MutL homologue) heterodimer which consists of the proteins hMLH1(human MutL homologue 1) and hMPMS2 (human homologue of yeast post meiotic segregation gene 2).

Unlike the previous examples given of nitrogen mustard resistance where enhanced repair correlates with a decrease in sensitivity, a defect in mismatch repair

results in resistance to DNA damaging agents. Two possible explanations have been put forward. In the first, the binding of the repair proteins to the DNA lesion may activate signal transduction pathways leading to cell cycle arrest and/or cell death (Hawn *et al.*, 1995; Nehme *et al.*, 1997). Secondly, it may be the case that futile cycles of synthesis past the DNA lesion, followed by removal of the newly synthesised strand increases the probability of generating a large gap or strand break in the DNA which eventually leads to the induction of cell death (Fink *et al.*, 1996; Vaisman *et al.*, 1998).

Following the *in vitro* selection of a human ovarian carcinoma cell line for cisplatin resistance, loss of mismatch repair activity arose through defects in the hMLH1 subunit. In addition, defects in cells with no prior exposure to cisplatin were linked to loss of the hMSH2 subunit (Aebi *et al.*, 1996). A number of topo II inhibiting agents such as doxorubicin and etoposide have also displayed low level resistance in mismatch repair deficient cell lines. Therefore, it has been suggested that MMR proteins may be involved in the detection of cleavable complexes and thus act to promote DNA damage (Drummond *et al.*, 1996). In contrast, no change in sensitivity was observed with the bifunctional nitrogen mustard melphalan suggesting that DNA adducts produced by these agents are not recognised by the MMR protein complex (Aebi *et al.*, 1997).

The development of cross-resistance between anti-tumour agents reduces the potential for maintaining relevant levels of chemotherapy. This is exemplified by the agents mitoxantrone and doxorubicin where resistance associated with either one of these agents tends to result in cross-resistance with the other. The development of multi-factorial resistance can lead to reduced sensitivity to drugs with completely different spectrums of activity and eventually hinders their use in combination therapy. In respect of this, the search for compounds that display good anti-tumour activity without the detrimental effects of MDR continues.

1.6 TOXICITY AND RESISTANCE MODULATORS

The resistance of cancer cells to chemotherapeutic agents can be caused by a number of mechanisms which result in the poor outcome of treatment. In an attempt to

overcome resistance, research has focused on the development of drugs that selectively inhibit the intracellular targets associated with resistance and hence potentiate the cytotoxic action of anti-cancer agents.

One of the first resistance modulators to undergo trials against the *MDR1* gene product Pgp, was the calcium antagonist verapamil. The rationale behind the use of calcium channel inhibitors as modulators of resistance was that drug efflux is likely to be related to calcium transport across cellular membranes and therefore modulation of transport proteins within the membrane would also result in modulation of resistance. Although the mechanism of action of verapamil is not fully defined, it has been shown to inhibit Pgp by increasing its phosphorylated state (Endicott *et al.*, 1989; Yusa *et al.*, 1989). A number of groups have demonstrated that verapamil is an effective agent against anthracycline resistance. For example, in CHrC5, a resistant CHO cell line overexpressing Pgp, verapamil induced the redistribution of doxorubicin from the cytoplasm to the nucleus enhancing its cellular accumulation and restoring the cytotoxicity of the drug (Bellamy *et al.*, 1988, Yusa *et al.*, 1989). However, despite its early *in vitro* success, trials of verapamil with doxorubicin against advanced epithelial ovarian cancer demonstrated that the equivalent non-cytotoxic concentration required to inhibit resistance *in vitro* was toxic in plasma levels *in vivo*. As a result verapamil became less attractive as a resistance inhibitor because of its cardiotoxic potential at the *in vivo* inhibitory concentration (Echizen *et al.*, 1985). More recently the D-isomer of verapamil (D-verapamil) which is 3-4 fold less cardiotoxic but is equipotent to racemic verapamil has shown good response in pancreatic cancer and it is currently undergoing clinical trials (Kornek *et al.*, 1995). Another example of a MDR modulator is Cyclosporine A a drug that functions to inhibit more than one mechanism of resistance. These include the inhibition of calmodulin, Pgp and the lowering of GSH levels (Twentyman, 1988).

The development of inhibitors against alkylating agent resistance has mainly focused on identifying drugs that exert their action by affecting drug detoxifying conjugation. Examples of two such inhibitors are butathionine sulfoximine (BSO) and ethacrynic acid (ER). The drug BSO functions to irreversibly inhibit the enzyme γ -glutamyl cysteine synthetase (γ -GCS) and thus decreases intracellular glutathione levels.

Consequently a reduced concentration of drug-GSH conjugates is formed and exported from the cell. The administration of BSO in combination with melphalan to patients with various cancers showed a good reduction in GSH levels with acceptable toxicity. In view of favourable results it is currently undergoing phase I clinical trials (O' Dwyer *et al.*, 1992 and 1996). The diuretic ethacrynic acid reversibly inhibits the GST isoenzymes and serves to prevent enzyme catalysed drug conjugation with GSH (Ahokas *et al.*, 1985; Giaccio *et al.*, 1991). It has also shown effectiveness in phase I trials (O'Dwyer *et al.*, 1991).

Although many chemical modulators have proved effective *in vitro*, they have yet to show a significant reversal of MDR in the clinic. The poor response is partly related to the toxicity of these agents and also to the existence of more than one mechanism of resistance in cancer cells. Recently, a completely different approach to toxicity and resistance modulation has come to the forefront and this involves the use drug-containing liposomes. These microscopic capsules (<100nm) are composed of a lipid bilayer membrane enclosing an active drug within a central aqueous compartment (Kim *et al.*, 1993). By undergoing slow degradation the goal of the liposomes is to alter the tissue distribution and pharmacokinetics of the drug, improving its efficacy and reducing its toxicity. Liposomes have no affinity for tumour cells but sequester within the tumour microvasculature slowly releasing drug into the immediate environment (Gabizon *et al.*, 1994). Because they take longer than non-encapsulated drug to eliminate from the body an increased concentration of drug is found at the target site. This was exemplified by doxorubicin and mitoxantrone containing liposomes which have both demonstrated prolonged cytotoxicity *in vitro* (Law *et al.*, 1996; Wiles *et al.*, 1997) and *in vivo* (Chang *et al.*, 1997). Hence, the positive evidence following the use of liposomal drug increases the prospect for higher doses of drug to be administered during treatment which should lead to a more effective clinical outcome with reduced toxic side-effects.

1. 7. AIMS

The previous work carried out concerning the anthraquinone DNA intercalating agents and bifunctional nitrogen mustard anti-cancer drugs has been summarised in the introduction (sections 1. 3 and 1. 4). From the information gathered it may be concluded that in order to achieve effective anti-tumour activity it is necessary to identify drugs that target and bind to their biological goals more efficiently. Such compounds could potentially improve drug potency and circumvent the problems associated with resistance (section 1. 5). Several attempts have been made to develop intercalating agents that bind to DNA covalently and irreversibly inhibit the topo II cleavable complex, an important cellular target of many anticancer drugs. Much of this previous work has focused on the synthesis of aliphatic and aniline nitrogen mustards linked to an acridine chromophore, an efficient intercalator (see section 1. 4. 4. 1). The acridine mustards have demonstrated a considerable increase in potency over non-targeted mustards and can therefore be considered as a basis for further synthetic development. The long established anti-tumour drug mitoxantrone a known topo II inhibitor, is one of the most effective drugs in clinical use today (see section 1. 3. 2. 1). It comprises of a planar tri-cyclic aromatic chromophore substituted on the 1,4 position with two basic alkylamino side-chains. The ease of manipulation of the sidechains and the aromatic chromophore of the anthraquinones makes them ideal candidates for drug development as DNA targeted alkylating agents.

The aims of this study are, therefore, to investigate the hypothesis that alkylating anthraquinones can irreversibly inhibit topo II and evade the mechanisms of resistance associated with both intercalating and alkylating anticancer agents. This will be achieved by:-

[1] Synthesizing a number compounds consisting of an anthraquinone chromophore substituted with at least one alkylating alkylamino side chain.

- [2] Determining the covalent binding interactions of selected alkylating anthraquinones with DNA, in particular, their nucleotide sequence specificity and ability to crosslink DNA.
- [3] Comparing the intercalating ability and cytotoxicity *in vitro* of the alkylating anthraquinones to their non-alkylating anthraquinone analogues in resistant cell lines.
- [4] Providing information concerning the effect of a selected number of alkylating and non-alkylating anthraquinone analogues on human topo II α and β dependent yeast *Saccharomyces cerevisiae*.

Chapter 2 SYNTHESIS AND ANALYSIS OF SUBSTITUTED ANTHRAQUINONES

2.1 INTRODUCTION

The nitrogen mustards are a family of highly reactive antineoplastic agents which covalently bind with nucleophiles. Although DNA is their primary cellular target, their lack of specificity for it leads to diminished drug cytotoxicity and additional toxic side effects (see section 1. 4). In view of this, a number of groups have explored the potential for enhancing the specificity of the nitrogen mustards by combining their structure with a DNA affinic chromophore such as acridine. More significantly, when comparing non-targeted with targeted mustards, the increased cytotoxicity of the latter was determined to be a consequence of enhanced target selection (see section 1. 4. 4).

The anthraquinone chromophore was chosen as the basis for the synthesis of the following DNA directed aliphatic nitrogen mustards as it is an integral constituent of a number of highly DNA affinic drugs such as mitoxantrone and AQ4, which exert their cytotoxicity through the inhibition of the enzyme topo II (see section 1. 2. 3). It is envisaged that a drug which comprises of an anthraquinone chromophore substituted with alkylating alkylamino sidechain(s) would have both DNA specificity and covalent binding ability.

By utilising an anthraquinone chromophore and altering side chain length and terminal substituent composition a series of mono substituted anthraquinones, 1,4 di-substituted 5,8-dihydroanthraquinones (ametantrone analogues) and 5,8-dihydroxyanthraquinones (mitoxantrone analogues) with mono and bis-alkylating side chains were synthesised. Furthermore, by making the terminal nitrogen on each side chain tertiary it was anticipated that synthesis of an N-oxide prodrug might be feasible.

2. 2 CHEMICALS AND REAGENTS

All reagents purchased were of a minimum 95% purity. 1-chloroanthraquinone, benzophenone, lithium aluminium hydride, bromoacetonitrile, bromoacrylonitrile, *N*-2-hydroxyethyl-*N*-methylamine, *N*-methylaminoethylenediamine, *N*-phenylaminoethylenediamine, boron trifluoride etherate, *N,N*-dimethylethylenediamine, thionyl chloride, triphenylphosphine, trifluoro acetic acid and 2-methoxyethanol were all supplied by Sigma-Aldrich, Poole, Dorset. 2, 3-dihydroleucoquinizarin from Pfaltz and Bauer, UK. Ethylene oxide was supplied by Fluka Chemicals, Gillingham, Dorset. *N,N*-2-hydroxyethylpropylenediamine obtained from Tokyo Kasei Chemicals, Hadfield, Derbyshire. Metachloroperoxybenzoic acid was obtained from Aldrich Rare Chemicals, USA. Leuco-1,4,5,8-tetrahydroxyanthraquinone was prepared from the method of (Greenhalgh *et al.*, 1968) by Ketan Ruparelia, Pharmacy Dept, De Montfort University, Leicester. Chromatography column silica: particle size 35-70 μ and 20-35 μ , was supplied by Fisher Scientific, Loughborough, England. Thin layer chromatography plates (Aluminium backed) supplied by Merck, Poole, Dorset, England. Reverse phase C8 Hplc column supplied by Bondapack, UK.

All solvents were of analytical (HPLC) grade: Dichloromethane, Carbon tetrachloride, Chloroform, Ethanol, Methanol, Diethylether, Hexane, Toluene, Ethylacetate and Acetonitrile were supplied by Fisher Scientific, Loughborough, England.

2. 3 SAMPLE ANALYSIS

The UV/Vis absorbance of the drugs was recorded on a Beckman DU70 UV/Vis Spectrophotometer fitted with deuterium and tungsten lamps. The infra-red absorbance was recorded on a Nicolet 205 FT-IR Spectrometer. Proton NMR spectra were reproduced on a Bruker AC 250 MHz Nuclear Magnetic Resonance Spectrometer, by Dr M.Needham, Department of Chemistry, De Montfort University, Leicester. Fast Atom Bombardment (FAB+) Mass-Spectra sample identification was obtained on a V.G 70 SEQ Mass Spectrometer (Manchester, England) by J. Lamb, MRC Toxicology Unit, Leicester

University, UK. HPLC analysis of ZP1 was performed on a Waters 501 twin pump system linked to a gradient controller. The drug was eluted over 30 minutes under gradient control at a flow rate of 1ml/min. The mobile phase gradient was raised from 100% dH₂O (0.1% TFA) at the start of analysis to 100% Methanol (0.1% TFA) after 30 minutes sample elution. Sample detection took place through a Waters 410 UV/Vis Spectrophotometer at a wavelength of 254nm and all data was recorded on Millenium software.

2. 4 EXPERIMENTAL

2. 4. 1. SIDE CHAIN PREPARATION

(1) *N*-(2-Hydroxyethyl)-*N*-methylethylenediamine (Kohn *et al.*, 1994)*

a) [N-(2-hydroxyethyl)-N-methylamino]acetonitrile. 2-Methylaminoethanol (10cm³, 0.125mol) was warmed under nitrogen at a temperature between 40-50°C. Bromoacetonitrile (4.0cm³, 0.05mol) was added dropwise to the flask and the reactants stirred vigorously over a period of 1 hour. The temperature of the reaction was then elevated to 110°C and the mixture allowed to stir for a further 2 hours. On cooling, water (20cm³) was added and the mixture extracted into dichloromethane (3×20cm³). The combined organic layers were dried over sodium sulphate and the solvent removed under reduced pressure. Distillation by Kugelrohr at 85-95°C/5mmHg afforded the product impure as a colourless viscous oil (3.67g, 64.1% yield). TLC (MeOH/CH₂Cl₂ 1:5; R_f 0. 6); Lit values*: ¹H NMR (CDCl₃) δ: 2.25 (s, 3H, CH₃N), 2.5 (t, 2H, CH₂CN), 2.58 (t, 2H, CH₂N), 3.6 (t, 2H, CH₂OH).

The product was used without further purification in the following procedure.

b) N-(2-Hydroxyethyl)-N-methylethylenediamine To a cooled suspension of lithium aluminium hydride (5.7g, 0.15mol) in dry tetrahydrofuran (150cm³) was slowly added [*N*-(2-hydroxyethyl)-*N*-methylamino]acetonitrile (5.72g, 0.05mol) in dry tetrahydrofuran (5.0cm³). The mixture was stirred for 1 hour at 0°C and then refluxed for a further 2.5

hours. After the reaction mixture had cooled, the following were added in succession; ethyl acetate (20cm³), distilled water (0.57cm³), 15% sodium hydroxide solution (0.57cm³) and distilled water (1.71ml). The mixture was then filtered under vacuum to remove the inorganic salts formed, the solvents removed under reduced pressure and the resulting filtrate partitioned between distilled water (100cm³) and chloroform (3×100cm³). Distillation by Kugelrohr at 95-105°C/5mmHg afforded the product as a golden viscous oil of approximately 80 % purity (3.5g, 59.3% yield). TLC (MeOH/CH₂Cl₂ 1:5; R_f 0.1); ¹H NMR (CDCl₃) δ: 2.3 (s, 3H, CH₃N), 2.5(t, 2H,CH₂N), 2.58 (t, 2H, CH₂N), 2.8 (t, 2H, CH₂NH₂), 2.81 (bs, 2H, NH₂CH₂), 3.6 (t, 2H, CH₂OH).

(2) *N*-(2-Hydroxyethyl)-*N*-methylpropanediamine (Kohn *et al.*, 1994).

a) [N-(2-Hydroxyethyl)-N-methyl-amino]acrylonitrile The method and conditions employed in the synthesis of this compound follow the procedure for the synthesis of [*N*-(2-hydroxyethyl)-*N*-methylamino]acrylonitrile. The concentration of reactants used were; 2-methylaminoethanol (11.4cm³, 0.14mol), acrylonitrile (6.2cm³, 0.094mol). Kugelrohr distillation at 115-120°C/ 0.25mmHg afforded a clear viscous oil (5.57g, 46.3% yield). TLC (MeOH/CH₂Cl₂ 1:5; R_f 0. 7); ¹H NMR (CDCl₃) δ: 2.28 (s, 3H, CH₃N), 2.54 (t, 2H, CH₂CN), 2.65 (t, 4H,CH₂N), 2.8 (t, 2H,CH₂N), 3.65 (t, 2H, CH₂OH).

The product was used without further purification in the following procedure.

b) N-(2-Hydroxyethyl)-N-methylpropanediamine. The reaction procedure and conditions follow those employed in the synthesis of *N*-(2-hydroxyethyl)-*N*-methylethylenediamine. Concentration of reactants used were; [*N*-(2-hydroxyethyl)-*N*-methylamino]acrylonitrile (5.57g, 0.049mol), lithium aluminium hydride (5.55g, 0.146mol). Distillation by Kugelrohr at 160-165°C, 0.25 mmHg afforded the product as a golden viscous oil of 80% purity (4.8g, 74.2% yield). TLC (MeOH/CH₂Cl₂ 1:5; R_f 0. 15); ¹H NMR (D₂O) δ: 1.8 (m, 2H, CH₂CH₂CH₂), 2.28 (s, 3H, CH₃N), 2.4 (t, 2H, CH₂NH₂), 2.58 (t, 4H,CH₂N), 3.62 (t, 2H, CH₂OH).

(3) *N,N*-Bis(2-hydroxyethyl)ethylenediamine

a) *Monoacetyethylenediamine* (Aspinall *et al.*, 1949). Ethyl acetate (528.3g, 6.3mol) and ethylenediamine (1550.0g, 25.8 mol) were stirred together at room temperature for several days until the mixture had become homogenous. The unreacted ethyl acetate was removed under reduced pressure and the resulting product was collected by high vacuum distillation at 115°C-130°C/5mmHg to give (272.4g, 44.6% yield). TLC (MeOH/NH₃ (4 drops); R_f 0.6);

The product was utilised in the next step of the reaction without identification or further purification.

b) *N-Acetyl-N,N-bis(2-hydroxyethyl)ethylenediamine*. (Peck *et al.*, 1961).

Monoacetyethylenediamine (150.5g, 1.5mol) was dissolved into methanol (200cm³) and cooled to 0°C by keeping the reaction flask on ice and by fixing a liquid nitrogen cold trap to the top of the condenser. To the flask was added ethylene oxide (194g, 4.4mol), the mixture stirred at 0°C for 2.5 hours, and then at room temperature for 18h once the cold trap had been removed. Ethylene oxide (97.0g, 2.2mol) was again added at 0°C using the same procedure and the mixture stirred at this temperature for 1.5 hours then at reflux for a further 6 hours. On cooling, the unreacted ethylacetate was removed under reduced pressure and the resulting product collected by high vacuum distillation at 220-230°C/1.25mmHg, to give (265.2g, 52.3% yield). TLC (MeOH/CH₂Cl₂, 1:1; R_f 0.5); ¹H NMR (CDCl₃) δ: 2.0 (bs, 3H, CH₃NCO), 2.6 (bt, 6H, 3×CH₂NH₂), 3.3 (bq, 2H, CH₂NH₂), 3.5 (t, 4H, 2CH₂OH), 3.7 (t, 1H, NHCO), 4.5 (bs, 1H, CH₂OH).

*The product was utilised in the next step of the reaction without further purification.

c) *N,N-bis(2-Hydroxyethyl)ethylenediamine*. To **N*-acetyl-*N,N*-bis(2-hydroxyethyl)-ethylenediamine (265.2g, 1.3mol) was added hydrochloric acid (5M, 180cm³) and the mixture refluxed for 6 hours. On cooling, the mixture was made basic to pH 8 with aqueous sodium hydroxide solution (1M), and the salt removed by precipitation with ethanol. All solvents were removed under reduced pressure and the final product collected at

approximately 80% purity by high vacuum distillation at 252-260°C/ 5mmHg to give (81.3g, 39.4% yield). TLC (MeOH/CH₂Cl₂ 1:1; R_f 0. 1); ¹H NMR (CD₃OD) δ: 2.5 (t, 2H, CH₂NH₂), 2.7 (t, 6H, 3CH₂N), 3.7 (t, 4H, 2CH₂OH).

(4) *N*-(2-Hydroxyethyl)-*N*-phenylethylenediamine

a) *N*-Phenylaminoethyl-*N'*-diphenylimine. To *N*-phenylethylenediamine (20.1g, 0.15mol) in xylene (50cm³) was added benzophenone (25.84g, 0.142mol) and a catalytic amount of boron trifluoride etherate (0.1ml). The reaction mixture was heated to reflux and the molar equivalent of water formed removed by Dean and Stark azeotropic distillation. On cooling, distilled water (2×50cm³) was added to extract the unreacted amine from the organic layer and following the removal of the organic solvent the product precipitated as a white crystalline solid (25.6g, 58.0% yield). TLC (EtOH/CH₂Cl₂ 1:5; R_f 0. 2).

The product was put forward to the next step of the reaction without identification or further purification.

b) *N*-(2-Hydroxyethyl)-*N*-phenylaminoethyl-*N'*-diphenylimine. To a cooled solution of *N*-phenylaminoethyl-*N'*-diphenylimine (25.6g, 0.08mol) in methanol (200cm³) was added ethylene oxide (11.3g, 0.25mol). The mixture was allowed to stir at 0°C for 2.5 hours and then at room temperature overnight. Following this, ethylene oxide (5.28g, 0.125mol) was again added to the mixture at 0°C for 1.5 hours and then the mixture further refluxed for 6 hours. On cooling, the solvent was removed and the product dried under vacuum to give a white crystalline solid (21.5g, 78% yield). TLC (EtOH/CH₂Cl₂ 1:5; R_f 0.7);

The product was put forward to the next step of the reaction without identification or further purification.

c) *N*-(2-Hydroxyethyl)-*N*-phenylethylenediamine. 2-[*N*-(2-hydroxyethyl)-*N*-phenylamino]-ethyl-*N*-diphenylimine (21.5g, 0.06mol) was added to a mixture of hydrochloric acid (2M, 200cm³) and methanol (50cm³), and allowed to stir at room temperature for 12 hours. Following the removal of the methanol under vacuum, dichloromethane (2×200cm³) was

added to separate the cleaved benzophenone from the mixture and the product, a hydrochloride, was extracted into the acidic water layer. The water layer was then made basic to pH 8 with sodium hydroxide (5M), the water removed, and the inorganic salts separated from the product by precipitation in ethanol. The product a golden/brown viscous oil (B.pt. 225-235°C/ 5mmHg) of approximately 80% purity was isolated on removal of solvent (10.3g, 91.3% yield). TLC (MeOH/CH₂Cl₂ 1:1; R_f 0.5); ¹H NMR (CD₃OD) δ: 3.2 (t, 2H, CH₂NH₂), 3.6(t, 2H, CH₂OH) 3.8 (t, 4H, CH₂N), 7.3 (t, 2H, Ar-*H*), 7.8 (t, 1H, Ar-*H*), 8.0 (d, 2H, Ar-*H*).

2. 4. 2 SYNTHESIS OF MONO SUBSTITUTED HYDROXYETHYL-AMINOANTHRAQUINONES

(5) 1-[*N*-[2-[*N*-(2-Hydroxyethyl)-*N*-methylamino]ethyl]amino]-9,10- anthracenedione (ZP1)

To *N*-(2-hydroxyethyl)-*N*-methylethylenediamine (2.4g, 0.02mol) in 2-methoxyethanol (10cm³) was added 1-chloroanthraquinone (0.5g, 2.05mmol) and the mixture refluxed for 6 hours. On evaporation of the solvents the resulting waxy solid was partitioned between dichloromethane (20cm³) and distilled water (3×20cm³) to remove any unreacted amine. The separated organic layer was removed *in vacuo* and the crude product lyophilised. Purification by flash column chromatography (CH₂Cl₂: EtOH, 9:1; TLC R_f 0.8) followed by precipitation from ethanol with dry diethylether gave on drying, the product as a red/orange powder (100mg, 15.3% yield). m.p.105.9 - 106.8°C; ¹H NMR (CDCl₃) δ: 2.3 (s, 3H, CH₃N), 2.7 (t, 2H, CH₂CH₂N), 2.8 (t, 2H, CH₂CH₂N), 3.4 (t, 2H, CH₂NHAr), 3.7 (t, 2H, CH₂OH), 7.0 (d, 1H, Ar-*H*), 7.5 (m, 2H, Ar-*H*), 7.7 (m, 2H, Ar-*H*), 8.2 (d, 1H, Ar-*H*), 8.3 (d, 1H, Ar-*H*), 10.1 (t (broad), 1H, NHAr); m/z FAB-MS (MeOH in 3-NBA) 325 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1650 (C=O), 1040 (N-H); E_λ (MeOH/DMSO) = 4761cm⁻¹; λ_{max} (521nm)

(6) 1-[N-[3-[N-(2-Hydroxyethyl)-N-methylamino]propyl]amino]-9,10-anthracenedione (ZP150)

For reaction procedure and conditions follow the method for the synthesis of ZP1. The reactants used were: 1-chloroanthraquinone (0.2g, 0.82mmol) and *N*-(2-hydroxyethyl)-*N*-methylpropanediamine (1.1g, 8.2mmol). Purification by flash column chromatography (CH₂Cl₂: EtOH, 9:1; TLC R_F 0.8) gave the product, a red powder (47.2mg, 17.5% yield). m.p. 98.5 - 99.2°C; ¹H NMR(CDCl₃) δ: 1.8 (m, 2H, CH₂CH₂CH₂), 2.3 (s, 3H, CH₃N), 2.7 (t, 2H, CH₂N), 2.8 (t, 2H, HNCH₂CH₂N), 3.4 (t, 2H, CH₂NHAr), 3.7 (t, 2H, CH₂OH), 7.0 (d, 1H, Ar-*H*), 7.5 (m, 2H, Ar-*H*), 7.7 (m, 2H, Ar-*H*), 8.2 (d, 1H, Ar-*H*), 8.3 (d, 1H, Ar-*H*), 10.1 (t(broad), 1H, NHAr); m/z FAB-MS (MeOH in 3-NBA) 325 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1650 (C=O), 1040 (N-H); E_λ (MeOH/DMSO) = 3514cm⁻¹; λ_{max} (504nm).

(7) 1-[N-[3-[N'-N''-Bis(2-hydroxyethyl)amino]propyl]amino]-9,10-anthracenedione (ZP233)

To *N,N*-bis(2-hydroxyethyl)propanediamine (3.3g, 20.0mmol) in 2-methoxyethanol (10cm³) was added 1-chloroanthraquinone (0.5g, 2.05mmol) and the mixture refluxed for 6 hours. On cooling the reaction mixture was partitioned between distilled water (3×20cm³) and dichloromethane (20cm³) to remove any unreacted amine, and the organic layer evaporated *in vacuo*. The product was dried under vacuum to give a solid shown by TLC (CH₂Cl₂: EtOH, 8:2; R_F 0.8) to contain unreacted 1-chloroanthraquinone (R_F 9.5). The unreacted 1-chloroanthraquinone was precipitated with methanol (10cm³) and the required product isolated from the filtrate on evaporation of the solvent. The product was further purified by precipitation in methanol with dry ethereal hydrogenchloride, to give a red powder (75.3mg, 10.2% yield). m.p. 91.2 - 92.2 °C. ¹H NMR (CDCl₃) peaks: δ 1.8 (m, 2H, CH₂CH₂CH₂), 2.3 (t, 6H, 3×CH₂N), 3.4 (t, 2H, CH₂NHAr), 3.7 (t, 4H, 2×CH₂OH), 7.0 (d, 1H, Ar*H*), 7.5 (m, 2H, Ar*H*), 7.7 (m, 2H, Ar*H*), 8.2 (d, 1H, Ar*H*), 8.3 (d, 1H, Ar*H*), 10.1 (t(broad), 2H, NHAr); m/z FAB-MS (MeOH in 3-NBA) 369 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹

3600-3300 (OH), 1650 (C=O), 1040 (N-H); E_{λ} (MeOH/DMSO) = 1961 cm^{-1} ; λ_{max} (510nm).

2. 4. 3. SYNTHESIS OF 1,4 DISUBSTITUTED-5,8-DIHYDRO-HYDROXYETHYLAMINOANTHRAQUINONES

(8) 1,4-Bis-[N-[2-[N'-(2-hydroxyethyl)-N'-methylamino]ethyl]amino]-9,10-anthracenedione (ZP242)

Under nitrogen, leucoquinizarin (0.3g, 1.26mmol) was added to warmed *N*-(2-hydroxyethyl)-*N*-methylethylenediamine (2.44g, 20.0mmol) and the reactants heated together at 60°C until TLC (MeOH; R_f 0.4) showed that the reaction had gone to completion (approximately 4 hours). Aqueous sodium hydroxide solution (15%, 0.2cm³) was then added to the cooled mixture and a brown/black to blue colour change was observed. In order to fully oxidize, the reaction was left to stir in air overnight. To remove the unreacted amine the mixture was partitioned between dichloromethane (80cm³) and distilled water (3×80cm³), the organic layer separated and the solvent evaporated *in vacuo* and the product dried under vacuum to give a waxy-solid. Purification by flash column chromatography (MeOH: CH₂Cl₂ 1:1; then MeOH). gave the product which was then further purified by precipitation from methanol with dry diethyl-ether to give dark blue/black crystals (105.7mg, 19.3% yield).m.p.181.2-182.3 °C; ¹H NMR (DMSO) peaks: δ 2.5 (bs, 6H, 2×CH₃N), 2.8 (bt, 2H, CH₂CH₂N), 3.0 (bt, 2H, CH₂CH₂N), 3.4 (bt, 2H, CH₂NHAr), 3.7 (bt, 2H, CH₂OH), 7.5 (s, 2H, Ar-*H*), 7.7 (m, 2H, Ar-*H*), 8.2 (m, 2H, Ar-*NH*), 10.7 (t (broad), 2H, 2×NHAr); m/z FAB-MS (MeOH in 3-NBA) 441 (M+H)⁺; IR ν_{max} (KBr) cm^{-1} 3600-3300 (OH), 1590 (C=O), 1230, (N-H); E_{λ} (dH₂O) = 7128 cm^{-1} ; λ_{max} (626nm)

(9) 1,4-Bis-[N-[3-[N',N'-bis(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione (ZP232)

Reaction conditions and procedure follow that for the synthesis of ZP242. The reactants used were *N,N*-bis(2-hydroxyethyl)ethylenediamine (3.7g, 20.0mmol) and

leucoquinizarin (0.3g, 1.26mmol). Purification by flash column chromatography (MeOH:CH₂Cl₂ 1:1; R_F 0.3) afforded the product as blue/black crystals (132.3mg, 21.8% yield). m.p. 177.5 - 178.9°C; ¹H NMR (DMSO) peaks: δ 3.4 (bt, 8H, 4×CH₂N), 3.5 (bt, 4H, 2×CH₂N), 3.85 (bt, 4H, 2×CH₂NHAr), 4.0 (bt, 8H, 4×CH₂OH), 7.7 (s, 2H, ArH), 7.85 (m, 2H, ArH), 8.3 (m, 2H, ArH), 10.4 (t(broad), 2H, 2×NHAr); m/z FAB-MS (MeOH in 3-NBA) 501 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1590 (C=O), 1230 (N-H); E_λ (dH₂O) = 8060 cm⁻¹; λ_{max} (624nm).

(10) 1,4-Bis-[N-[3-[N',N'-bis(2-hydroxyethyl)amino]propyl]amino]-9,10-anthracenedione (ZP240)

The reaction conditions and procedure follow that for the synthesis of ZP242. The reactants used were *N,N*-bis(2-hydroxyethyl)propanediamine (3.64g, 20.0mmol) and leucoquinizarin (0.3g, 1.26mmol). Purification by flash column chromatography (MeOH:CH₂Cl₂, 3:7 then increase to 1:1; R_F 0.4) afforded the product as blue/black crystals (113.0mg, 17.6% yield). m. p. 137.6 -138.6 °C; ¹H NMR (DMSO) peaks: δ 1.8 (m, 4H, 2×CH₂CH₂CH₂), 2.3 (bt, 8H, 4×CH₂N), 2.4 (bt, 4H, 2×CH₂N), 3.4 (bt, 4H, 2×CH₂NHAr), 3.7 (bt, 8H, 4×CH₂OH), 7.7 (s, 2H, ArH), 7.85 (m, 2H, ArH), 8.3 (m, 2H, ArH), 10.7 (t(broad), 2H, 2×NHAr); m/z FAB-MS (MeOH in 3-NBA) 529 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1590 (C=O), 1230 (N-H); E_λ (dH₂O) = 9964 cm⁻¹; λ_{max} (630nm).

(11) 1,4-Bis-[N-[2-[N⁷-(2-hydroxyethyl)-N⁷-phenylamino]ethyl]amino]-9,10-anthracenedione (ZP245)

The reaction conditions and procedure follow that for the synthesis of ZP242. The reactants used were *N*-(2-hydroxyethyl)-*N*-phenylethylenediamine (3.6g, 20.0mmol) and leucoquinizarin (0.3g, 1.26mmol). To aid solubility 2-methoxyethanol (10cm³) was employed as a reflux solvent during this reaction. Purification by flash column chromatography (EtOH: CH₂Cl₂: Hexane 0.2: 8: 1.8; R_F 0.45) afforded the product as a blue powder (195.5mg, 27.5% yield). TLC (EtOH:CH₂Cl₂, 0.1: 9.9; R_f 0.8); m.p 161.9 - 162.5 °C; ¹H NMR (DMSO) peaks: δ 3.35 (bt, 8H, 4×CH₂N), 3.5 (bt, 4H, 2×CH₂N), 3.6

(bt, 4H, 2×CH₂NHAr), 3.7 (bt, 8H, 4×CH₂OH), 6.6 (m, 2H, 2×ArH), 6.8 (m, 4H, 4×ArH), 7.2 (m, 4H, 4×ArH), 7.7 (s, 2H, ArH), 7.85 (m, 2H, ArH), 8.3 (m, 2H, ArH), 10.7 (t(broad), 2H, 2×NHAr); m/z FAB-MS (MeOH in 3-NBA) 529 (M+H)⁺; IR ν_{\max} (KBr) cm⁻¹ 3600-3300 (OH), 1590 (C=O), 1580, 1510 (Ar); 1230 (N-H); E_λ (MeOH/DMSO) = (13121); λ_{\max} (643nm).

2. 4. 4. SYNTHESIS OF 1,4 DISUBSTITUTED-5, 8-

DIHYDROXY-HYDROXYETHYLAMINOANTHRAQUINONES

(12) 1,4-Bis[*N*-[2-[*N'*-(2-hydroxyethyl)-*N'*-methylamino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione (ZP265)

Under nitrogen, leuco-1,4,5,8-tetrahydroxyanthraquinone (0.2g, 0.75mmol) was added to warmed *N*-(2-hydroxyethyl)-*N*-methylethylenediamine (1.3g, 12.0mmol) and the mixture heated together at 60°C for 5 hours. Following the addition of aqueous sodium hydroxide solution (2M, 0.2cm³) the mixture was exposed to air and further stirred at room temperature overnight. To remove impurities, flash column chromatography was performed (MeOH: CH₂Cl₂: NH₃, 0.5: 9.4: 0.1; TLC: R_F 0.25) and the column solvents removed *in vacuo*. The resulting pure product was precipitated from methanol with dry diethylether and dried under vacuum to give a dark blue powder (87.1mg, 24.6% yield). m. p. 211.3 - 213.5 °C; ¹H NMR(CDCl₃) δ : 2.3 (s, 6H, 2×CH₃N), 2.7 (t, 4H, 2×CH₂N), 2.8 (t, 4H, 2×CH₂N), 3.6 (t, 4H, 2×CH₂NAr), 3.7 (t, 4H, 2×CH₂OH), 7.2 (s, 2H, ArH), 7.4 (s, 2H, ArH), 10.6(t(broad), 2H, 2×NHArH); m/z FAB-MS (MeOH in 3-NBA) 474 (M+H)⁺; IR ν_{\max} (KBr) cm⁻¹ 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E_λ (dH₂O) = 14604 cm⁻¹; λ_{\max} (608nm).

(13) 1,4-Bis[*N*-[3-[*N'*-(2-hydroxyethyl)-*N'*-methylamino]propyl]amino]-5,8-dihydroxy-9,10-anthracenedione (ZP273)

The reaction procedure and conditions follow that for the synthesis of ZP265. The reactants used were; *N*-(2-hydroxyethyl)-*N*-methylpropanediamine (1.45g, 10.0mmol) and leuco-1,4,5,8-tetrahydroxyanthracenedione (0.2g, 0.75mmol). Purification by flash column

chromatography (MeOH: CH₂Cl₂: NH₃, 0.25: 9.75: 0.05; TLC R_F 0.5) afforded the product as a dark blue powder (45.1mg, 12.0% yield). m. p. 157 - 158 °C; ¹H NMR(CDCl₃) δ: 1.9 (m, 4H, 2×CH₂CH₂CH₂), 2.35 (s, 6H, 2×CH₃N), 2.7 (t, 4H, 2×CH₂N), 2.8 (t, 4H, 2×CH₂N), 3.5 (t, 4H, 2×CH₂NAr), 3.7 (t, 4H, 2×CH₂OH), 7.15 (s, 2H, ArH), 7.35 (s, 2H, ArH), 10.5 (t(broad), 2H, 2×NHArH); m/z FAB-MS (MeOH in 3-NBA) 502 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E_λ (MeOH/DMSO) = 3688 cm⁻¹; λ_{max} (676nm).

(14) 1,4-Bis[*N*-[2-[*N*',*N*'-bis(2-hydroxyethyl)amino]ethyl]amino-5,8-dihydroxy-9,10-anthracenedione (ZP274)

The reaction procedure and conditions follow that for the synthesis of ZP265. The reactants used were; *N,N*-bis(2-hydroxyethyl)ethylenediamine (2.0g, 10.0mmol) and leuco-1,4,5,8-tetrahydroxyanthracenedione (0.2g, 0.75mmol). Purification by flash column chromatography (MeOH: 0.5% NH₃; TLC R_F 0.25) afforded the product as a dark blue powder (94.2mg, 23.6% yield); m. p. 218.5 - 219.5 °C; ¹H NMR(CDCl₃) δ: 2.7 (t, 8H, 4×CH₂N), 2.9 (t, 4H, 2×CH₂N), 3.5 (t, 4H, 2×CH₂NAr), 3.7 (t, 8H, 4×CH₂OH), 7.1 (s, 2H, ArH), 7.3 (s, 2H, ArH), 10.6 (t(broad), 2H, 2×NHAr); m/z FAB-MS (MeOH in 3-NBA) 533 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E_λ (dH₂O) = 8876 cm⁻¹; λ_{max} (606nm).

(15) 1,4-Bis[*N*-[3[*N*',*N*'-bis(2-hydroxyethyl)amino]propyl]amino]-5,8-dihydroxy-9,10-anthracenedione dihydrochloride (ZP255)

The reaction procedure and conditions follow that for the synthesis of ZP265. The reactants used were; *N,N*-bis(2-hydroxyethyl)propanediamine (2.16g, 10.0mmol) and leuco-1,4,5,8-tetrahydroxyanthracenedione (0.2g, 0.75mmol). Purification by flash column chromatography (MeOH: CH₂Cl₂: NH₃, 0.5: 9.5: 5drops; TLC R_F 0.25) afforded the product as a dark blue powder (128.1mg, 30.5% yield). m. p. 243.4 - 245.2 °C; ¹H NMR(DMSO) δ: 1.75 (bt, 4H, 2× CH₂CH₂CH₂), 2.5 (bt, 8H, 4×CH₂N), 2.6 (bt, 4H, 2×CH₂N), 3.45 (bt, 4H, 2×CH₂NAr), 3.6 (bt, 8H, 4×CH₂OH), 7.2 (s, 2H, ArH), 7.6 (s, 2H, ArH), 10.6 (t(broad),

2H, 2×NHAr); m/z FAB-MS (MeOH in 3-NBA) 561 (M+H)⁺; IR ν_{\max} (KBr) cm⁻¹ 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E_λ (dH₂O) = 9844 cm⁻¹; λ_{\max} (612nm).

(16) 1,4-Bis[*N*-[2-[*N'*-(2-hydroxyethyl)-*N'*-phenylamino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione (ZP254)

The reaction procedure and conditions follow that for the synthesis of ZP265 the exception being that the reaction mixture was heated at 100°C for 5 hours. The reactants used were: *N*-(2-hydroxyethyl)-*N*-phenylethylenediamine (1.98g, 10.0mmol) and leuco-1,4,5,8-tetrahydroxyanthracenedione (0.2g, 0.75mmol). Following purification by column chromatography (EtoAc: Hexane, 7.5: 2.5 increasing to 100% EtoAc; TLC R_F 0.8) the product was washed in diethylether and filtered to give a deep green/blue powder (74.6mg, 16.7% yield). m. p. 178.5 - 179.5 °C; ¹H NMR (DMSO) peaks: δ 3.35 (bt, 8H, 4×CH₂N), 3.5 (bt, 4H, 2×CH₂N), 3.6 (bt, 4H, 2×CH₂NHAr), 3.7 (bt, 8H, 4×CH₂OH), 6.65 (m, 2H, 2×ArH), 6.8 (m, 4H, 4×ArH), 7.2 (m, 4H, 4×ArH), 7.2 (s, 2H, ArH), 7.45 (s, 2H, ArH), 10.7 (t(broad), 2H, 2×NHAr); m/z FAB-MS (MeOH in 3-NBA) 596 (M+H)⁺; IR ν_{\max} (KBr) cm⁻¹ 3600-3300 (OH), 1590 (C=O), 1600, 1510 (Ar); 1230 (N-H); E_λ (MeOH/DMSO) = 15319 cm⁻¹; λ_{\max} (681nm).

2. 4. 5. SYNTHESIS OF UNSYMMETRICAL 1, 4-DISUBSTITUTED-5,8 DIHYDROXY HYDROXYETHYLAMINOANTHRAQUINONES

(17) 1-[*N*-[2-[*N'*-(2-Hydroxyethyl)-*N'*-methylamino]ethyl]amino]-4-[*N''*-[2-[*N'''*,*N'''*-dimethylamino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione (ZP257)

Under nitrogen, leuco-1,4,5,8-tetrahydroxyanthraquinone (0.2g, 0.75mmol) was added to warmed *N*-(2-hydroxyethyl)-*N*-methylethylenediamine (0.71g, 6.0mmol) and the mixture allowed to stir for 30min. To this was then added *N,N*-(dimethyl)ethylenediamine (0.194g, 2.2mmol) and the reaction allowed to proceed at 80°C for 5 hours. After cooling, aqueous sodium hydroxide solution (15%, 0.2cm³) was added and the reaction exposed to air and left to stir overnight at room temperature. The crude product was identified by TLC,

isolated by flash column chromatography (MeOH: CH₂Cl₂: NH₃, 0.5: 9.5: 0.1; TLC (MeOH: CH₂Cl₂) R_F 0.35) and, following the removal of the solvent *in vacuo*, lyophilised to remove traces of water. Precipitation from methanol with dry diethylether and removal of trace solvent under vacuum gave a dark-blue powder (34.8mg, 10.5% yield). m. p. 207.5 - 209.5 °C; ¹H NMR(CDCl₃) δ: 2.7 (s, 9H, 3×NCH₃), 3.1 (t, 4H, 2×CH₂N), 3.3 (t, 2H, CH₂N), 3.9 (t, 4H, 2×CH₂NHAr), 4.1 (t, 2H, CH₂OH), 7.1 (s, 2H, ArH), 7.3 (s, 2H, ArH), 10.8 (t(broad), 2×H, NHAr); m/z FAB-MS (MeOH in 3-NBA) 443.2 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E_λ = 7128 cm⁻¹; λ_{max} (608nm).

(18) 1-[N-[2-[N',N'-Bis(2-hydroxyethyl)amino]ethyl]amino]-4-[N''-[2-[N''',N'''-dimethylamino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione (ZP275)

The reaction conditions and procedure follow that for the synthesis of ZP257. The reactants used were; *N,N*-bis(2-hydroxyethyl)ethylenediamine (0.67g, 3.6mmol), *N,N*-(dimethyl)ethylenediamine (0.194g, 2.2mmol) and leuco-1,4,5,8-tetrahydroxyanthraquinone (0.2g, 0.75mmol). Following purification by column chromatography (MeOH: CH₂Cl₂ : NH₃, 0.5: 9.5: 0.1 increasing to 2: 8: 0.1; TLC R_F 0.3) the product was isolated as a dark blue powder (60.03mg, 19.5% yield). m.p. 204.2-205.0°C; ¹H NMR(CDCl₃/CD₃OD) δ: 2.4 (s, 6H, 2×NCH₃), 2.9 (t, 4H, 2×NCH₂), 3.0 (t, 2H, CH₂N), 3.6 (t, 4H, 2×CH₂NHAr), 3.7 (t, 4H, 2×CH₂OH), 7.1(s, 2H, ArH), 7.3 (s, 2H, ArH), 10.6 (t(broad), 2×H, NHAr); m/z FAB-MS (MeOH in 3-NBA) 473 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E_λ = 7936 cm⁻¹; λ_{max} (608nm)

2. 4. 6. SYNTHESIS OF CHLOROETHYLAMINOANTHRAQUINONES

(19) 1-[N-[2-[N-(2-Chloroethyl)-N-methylamino]ethyl]amino]-9,10-anthracenedione hydrochloride (ZP293)

Under nitrogen, triphenylphosphine (0.16g, 0.6mmol) then carbon tetrachloride (0.28g, 1.8mmol) was added to a stirred solution of ZP1 (0.1g, 0.31mmol) in dichloromethane (5.0cm³) and the suspension allowed to stir at room temperature for 24 hours. The crude product was precipitated by the addition of dry ethereal hydrogen chloride

and the precipitate filtered and dried under vacuum. To remove unreacted triphenylphosphine, the product was dissolved into the minimum amount of dichloromethane/methanol (1:1) then precipitated by the addition of an ethanol/ethylacetate mixture (1:1) at 60°C and dried under vacuum. The product was isolated as a red powder (93.5mg, 80% yield). TLC (EtOH: CH₂Cl₂, 0.5: 9.5; R_f 0.8); m. p. 207.1 - 209.2 °C; ¹H NMR (MeOD/CDCl₃) δ: 3.1 (s, 3H, CH₃N), 3.5 (bt, 2H, CH₂CH₂N), 3.7 (bt, 2H, CH₂CH₂N), 3.9 (t, 2H, CH₂NHAr), 3.95 (t, 2H, CH₂Cl), 7.3 (d, 1H, Ar-*H*), 7.7 (m, 2H, Ar-*H*), 7.8 (m, 2H, Ar-*H*), 8.2 (d, 1H, Ar-*H*), 8.3 (d, 1H, Ar-*H*), 10.1 (t (broad), 1H, NHAr); m/z FAB-MS (MeOH in 3-NBA) 343 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3550-3310 (NRH), 1600 (C=O), 1230 (N-H); E_λ (MeOH/DMSO) = 6963 cm⁻¹; λ_{max} (488nm).

(20) 1-[N-[3-[N-(2-Chloroethyl)-N-methylamino]propyl]amino]-9,10-anthracenedione hydrochloride (ZP288)

The reaction procedure and conditions follow that for the synthesis of ZP293. The reagents used were; ZP150 (0.05g, 0.15mmol), triphenylphosphine (0.078g, 0.3mmol) and carbon tetrachloride (0.137g, 0.9mmol). The product was isolated as a red powder (35.5mg, 60.2%). TLC (EtOH: CH₂Cl₂, 0.5: 9.5; R_f 0.9); m. p. 205 - 207 °C; ¹H NMR (MeOD) δ: 2.25 (m, 2H, CH₂CH₂CH₂), 3.1 (s, 3H, CH₃N), 3.4 (t, 2H, CH₂CH₂N), 3.54 (t, 2H, CH₂CH₂N), 3.7 (t, 2H, CH₂NHAr), 4.0 (t, 2H, CH₂Cl), 7.3 (d, 1H, Ar-*H*), 7.7 (m, 2H, Ar-*H*), 7.8 (m, 2H, Ar-*H*), 8.2 (d, 1H, Ar-*H*), 8.3 (d, 1H, Ar-*H*), 10.1 (t (broad), 1H, NHAr); m/z FAB-MS (MeOH in 3-NBA) 357 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3550-3310 (NRH), 1600 (C=O), 1230 (N-H); E_λ (MeOH/DMSO) = 1297.5 cm⁻¹; λ_{max} (498nm).

(21) 1-[N-[3-[N,N-Bis(2-chloroethyl)amino]propyl]amino]-9,10-anthracenedione hydrochloride (ZP284)

The reaction procedure and conditions follow that for the synthesis of ZP293. The reagents used were; ZP233 (0.05g, 0.136mmol); triphenylphosphine (0.071g, 0.27mmol) and carbon tetrachloride (0.246g, 1.62mmol). The product was isolated as a red powder (48.0mg, 80.3% yield). TLC (EtOH: CH₂Cl₂, 0.5: 9.5; R_f 0.8); m. p. 194.5 - 196.5 °C; ¹H

NMR (MeOD) δ : 2.2 (m, 2H, CH₂CH₂CH₂), 3.5 (t, 2H, CH₂CH₂N), 3.54 (t, 2H, CH₂CH₂N), 3.65 (t, 2H, CH₂CH₂N), 3.7 (t, 2H, CH₂NHAr), 4.0 (t, 4H, 2×CH₂Cl), 7.3 (d, 1H, Ar-H), 7.7 (m, 2H, Ar-H), 7.8 (m, 2H, Ar-H), 8.2 (d, 1H, Ar-H), 8.3 (d, 1H, Ar-H); m/z FAB-MS (MeOH in 3-NBA) 405 (M+H)⁺; IR ν_{\max} (KBr) cm⁻¹ 3550-3310 (NRH), 1600 (C=O), 1230 (N-H); E_λ (MeOH/DMSO) = 7948 cm⁻¹; λ_{\max} (503nm).

(22) 1,4-Bis[N-[2-[N'-(2-chloroethyl)-N'-methylamino]ethyl]amino]-9,10-anthracenedione dihydrochloride (ZP286)

Under nitrogen, triphenylphosphine (0.086g, 0.33mmol), then carbon tetrachloride (0.15g, 1.32mmol) were added to a stirred solution of ZP242 (0.05g, 0.11mmol) in a mixture of dichloromethane (4.0cm³) and acetonitrile (1.0cm³), and the suspension allowed to stir at room temperature for 48 hours. The crude product was precipitated by the addition of dry ethereal hydrogen chloride and following the removal of solvent traces under vacuum, triphenylphosphine impurities were removed by initially dissolving the compound into the minimum amount of dichloromethane/methanol (1:1) and then precipitating with an ethanol/ethylacetate mixture (1:1) at 60 °C. The product was isolated as a dark blue powder (47.4mg, 78.5% yield). TLC (EtOH: CH₂Cl₂, 1: 9; R_f 0.6); m. p. 185.4 - 186.0 °C; ¹H NMR (MeOD/CDCl₃) δ : 3.1 (s, 6H, CH₃N), 3.6 (bt, 4H, 2×CH₂CH₂N), 3.7 (bt, 4H, 2×CH₂CH₂N), 3.9 (t, 4H, 2×CH₂NHAr), 3.95 (t, 4H, 2×CH₂Cl), 7.6 (s, 2H, Ar-H), 7.8 (m, 2H, Ar-H), 8.3 (m, 2H, Ar-H), 10.1 (t (broad), 1H, NHAr); m/z FAB-MS (MeOH in 3-NBA) 477 (M+H)⁺; IR ν_{\max} (KBr) cm⁻¹ 3550-3310 (NRH), 1600 (C=O), 1230 (N-H); E_λ (MeOH/DMSO) = 12027 cm⁻¹; λ_{\max} (638nm).

(23) 1,4-Bis[N-[2-[N',N'-bis(2-chloroethyl)amino]ethyl]amino]-9,10-anthracenedione dihydrochloride (ZP290)

The reaction conditions and procedure follow that for the synthesis of ZP286. The reagents used were; ZP232 (0.05g, 0.09mmol), triphenylphosphine (0.144g, 0.5mmol) and carbon tetrachloride (0.32g, 2.1mmol). The product was isolated as a dark blue powder (35.4mg, 63.5% yield). TLC (EtOH: CH₂Cl₂, 1: 9; R_f 0.8); m. p. 194 -196°C; ¹H NMR

(MeOD/CDCl₃) δ : 3.6 (t, 4H, 2 \times CH₂CH₂N), 3.64 (t, 8H, 4 \times CH₂CH₂N), 3.9 (t, 4H, 2 \times CH₂NHAr), 4.0 (t, 8H, 4 \times CH₂Cl), 7.3 (d, 1H, Ar-*H*), 7.7 (m, 2H, Ar-*H*), 7.8 (m, 2H, Ar-*H*), 8.2 (d, 1H, Ar-*H*), 8.3 (d, 1H, Ar-*H*); *m/z* FAB-MS (MeOH in 3-NBA) 575 (M+H)⁺; IR ν_{\max} (KBr) cm⁻¹ 3550-3310 (NRH), 1600 (C=O), 1230 (N-H); E _{λ} (MeOH/DMSO) = 8570 cm⁻¹; λ_{\max} (638nm).

(24) 1,4-Bis[*N*-[3-[*N'*,*N'*-bis(2-chloroethyl)amino]propyl]amino]-9,10-anthracenedione dihydrochloride (ZP289)

The reaction conditions and procedure follow that for the synthesis of ZP286. The reagents used were; ZP240 (0.05g, 0.095mmol), triphenylphosphine (0.15g, 0.57mmol); and carbon tetrachloride (0.346g, 2.28mmol). The product was isolated as a dark blue powder (44.9mg, 70.1% yield). TLC (EtOH:CH₂Cl₂, 0.5:9.5; R_f 0.8); m. p. 187.2-188.9°C; ¹H NMR(CDCl₃/CD₃OD) δ : 2.2 (m, 4H, 2 \times CH₂CH₂CH₂), 3.5 (t, 4H, 2CH₂N), 3.6 (t, 8H, 4 \times CH₂N), 3.7 (t, 4H, CH₂NAr), 3.9 (t, 8H, 4 \times CH₂Cl), 7.4 (s, 2H, Ar*H*), 7.7 (m, 2H, Ar*H*), 8.2(m, 2H, Ar*H*); *m/z* FAB-MS (MeOH in 3-NBA) 603 (M+H)⁺; IR ν_{\max} (KBr) cm⁻¹ 3550-3310 (NRH), 1600 (C=O), 1230 (N-H); E _{λ} (MeOH/DMSO) = 2171 cm⁻¹; λ_{\max} (642nm).

(25) 1,4-Bis[*N*-[2-[*N'*-(2-chloroethyl)-*N'*-methylamino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione dihydrochloride (ZP285)

The reaction conditions and procedure follow that for the synthesis of ZP286. The reagents used were; ZP265 (0.05g, 0.11mmol); triphenylphosphine (0.08g, 0.32mmol) and carbon tetrachloride (0.19g, 1.2mmol). The product was isolated as a dark blue powder (52.5mg, 85.3% yield). m. p 201.2 - 203.1°C; TLC (EtOH: CH₂Cl₂, 0.5: 9.5; R_f 0.8); ¹H NMR (CDCl₃/CD₃OD) δ : 3.0 (s, 6H, 2 \times CH₃N), 3.5 (t, 4H, 2 \times CH₂NCH₂), 3.6 (t, 4H, 2 \times CH₂NCH₂), 3.9 (t, 4H, 2 \times CH₂NHAr), 3.9 (t, 4H, 2 \times CH₂Cl), 7.2 (s, 2H, Ar*H*), 7.4 (s, 2H, Ar*H*), 10.6 (t(broad), 2H, 2 \times NHAr); *m/z* FAB-MS (MeOH in 3-NBA) 509 (M+H)⁺; IR ν_{\max} (KBr) cm⁻¹ 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E _{λ} (MeOH/DMSO) = 27026 cm⁻¹; λ_{\max} (666nm).

(26) 1,4-Bis[*N*-[2-[*N'*,*N'*-bis(2-chloroethyl)amino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione dihydrochloride (ZP280)

The reaction conditions and procedure follow that for the synthesis of ZP286. The reagents used were; ZP274 (0.05g, 0.09mmol), triphenylphosphine (0.147g, 0.56mmol) and carbontetrachloride (0.34g, 2.25mmol). The product was isolated as a dark blue powder (45.6mg, 75.3% yield). TLC (EtOH: CH₂Cl₂, 0.5: 9.5, R_f 0.85); m. p. 191.6 -192.0°C; ¹H NMR (CDCl₃) δ 3.0 (m, 12H, 6×CH₂N), 3.5 (t, 4H, 2×CH₂NHAr), 3.7 (t, 8H, 2×CH₂Cl), 7.1 (s, 2H, ArH), 7.3 (s, 2H, ArH), 10.4 (t(broad), 2H, 2×NHAr); m/z FAB-MS (MeOH in 3-NBA) 607 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E_λ (MeOH/DMSO) = 8950 cm⁻¹; λ_{max} (673nm).

(27) 1,4-Bis[*N*-[3-[*N'*,*N'*-bis(2-chloroethyl)amino]propyl]amino]-5,8-dihydroxy-9,10-anthracenedione dihydrochloride (ZP282)

The reaction conditions and procedure follow that for the synthesis of ZP286. The reagents used were; ZP255 (0.05g, 0.79mmol), triphenylphosphine (0.124g, 0.47mmol) and carbon tetrachloride (0.288g, 1.89mmol). The product was isolated as a dark blue powder (44.7mg, 80.7% yield); TLC (EtOH:CH₂Cl₂ , 0.5:9.5) R_f 0.85; m. p. 205.1 -206.5°C; ¹H NMR (CDCl₃/CD₃OD) δ: 2.2 (t, 4H, 2×CH₂CH₂CH₂), 3.5 (t, 8H, 4×CH₂N), 3.6 (t, 4H, 2×CH₂N), 3.7 (t, 4H, 2×CH₂NHAr), 3.9 (t, 8H, 4×CH₂Cl), 7.1 (s, 2H, ArH), 7.3 (s, 2H, ArH), 10.6 (t(broad), 2H, 2×NHAr); m/z FAB-MS (MeOH in NBA) 635 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E_λ (MeOH/DMSO) = 9125 cm⁻¹; λ_{max} (675nm)

(28) 1-[*N*-[2-[*N'*,*N'*-Bis(2-chloroethyl)amino]ethyl]amino]-4-[*N''*]-[2-[*N'''*]-dimethylamino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione dihydrochloride (ZP281)

The reaction conditions and procedure follow that for the synthesis of ZP286. ZP275 (0.05g, 0.10mmol); triphenylphosphine (0.11g, 0.32mmol) and carbon tetrachloride (0.19g, 1.25mmol). The product was isolated as a dark blue powder (51.5mg, 83.2%); TLC

(EtOH: CH₂Cl₂, 0.5: 9.5; R_f 0.75); m. p. 190.0 -192.1°C; ¹H NMR(CDCl₃/CD₃OD) δ: 3.0(s, 6H, 2×NCH₃), 3.4 (t, 4H, 2×CH₂N), 3.6 (t, 4H, 2×CH₂N), 3.85 (t, 4H, 2×CH₂NHAr), 3.9 (t, 4H, 4×CH₂Cl), 7.1 (s, 2H, ArH), 7.3 (s, 2H, ArH), 10.6 (t(broad), 2H, 2×NHAr); m/z FAB-MS (MeOH in 3-NBA) 509 (M+H)⁺; IR ν_{max} (KBr) cm⁻¹ : 3600-3300 (OH), 1580 (C=O), 1230 (N-H); E_λ (MeOH/DMSO) = 14162 cm⁻¹; λ_{max} (620nm)

2. 4. 7 ATTEMPTED SYNTHESIS AND PURIFICATION OF SUBSTITUTED ANTHRAQUINONES

(29) 1,4-Bis[*N*-[2-[*N'*-(2-chloroethyl)-*N'*-phenylamino]ethyl]amino]-5,8-dihydroxy-9,10-anthracenedione dihydrochloride (ZP292)

The reaction conditions and procedure follow that for the synthesis of ZP286. The reagents used were; ZP254 (0.06g, 0.1mmol), triphenylphosphine (0.10g, 0.4mmol) and carbontetrachloride (0.182g, 1.2mmol). A product was isolated as a blue-green powder identified by NMR as cyclised and/or mono chlorinated product.

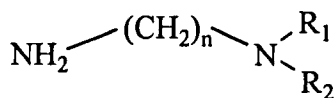
¹H NMR (CDCl₃) peaks: impure δ 3.6 (m, 8H, 4×CH₂N), 3.8 (m, 8H, 2×CH₂NAr; 2×CH₂Cl), 6.8 (m, 6H, 2×ArH), 7.1 (s, 2H, ArH), 7.2 (s, 2H, ArH), 7.3 (m, 6H, ArNH) 10.5 (t(broad), 2H, 2×NHAr).

(30) 1-[*N*-[2-[*N*-(2-Chloroethyl)-*N*-methylamino]ethyl]amino]-9,10-anthracenedione-N-oxide hydrochloride (ZP180)

To a solution of ZP293 hydrochloride (0.04g, 0.1mmol) in chloroform (3ml) at 0°C was added slowly a cooled solution of metachloroperoxybenzoic acid (mCPBA) (0.019g, 0.11mmol) in chloroform (1ml). After 1 hour TLC showed that the reaction had gone to completion (approximately 80% conversion) and the resulting mixture was then precipitated with dry ethereal hydrogenchloride to give the N-oxide hydrochloride. Following flash column chromatography (100% chloroform) on neutral alumina the product was isolated in solution as a single spot as shown by TLC. After the removal of the column solvent under reduced pressure at a temperature of 40°C, TLC showed that the compound had broken down to form an impurity. By washing with three aliquots of hexane some of

the impurity was removed, the product dried and isolated as red crystals of approximately 70% purity. ^1H NMR (DMSO) impure δ : 4.1 (s, 3H, CH_3N), 4.4 (bt, 4H, $2\times\text{CH}_2\text{CH}_2\text{N}$), 4.55 - 4.65 (bm, 4H, CH_2N and CH_2Cl), 7.8 (d, 1H, Ar-*H*), 7.9 (d, 1H, Ar-*H*), 8.1 (m, 1H, Ar-*H*), 7.8 (m, 2H, Ar-*H*), 8.2 (d, 1H, Ar-*H*), 8.3 (d, 1H, Ar-*H*), 10.1 (t (broad), 1H, NHAr); m/z FAB-MS (MeOH in 3-NBA) impure 359 ($\text{M}+\text{H}$) $^+$.

2.5. RESULTS



Side chain :-

- (1) $n = 2$, $\text{R}_1 = \text{CH}_2\text{CH}_2\text{OH}$, $\text{R}_2 = \text{CH}_3$
- (2) $n = 3$, $\text{R}_1 = \text{CH}_2\text{CH}_2\text{OH}$, $\text{R}_2 = \text{CH}_3$
- (3) $n = 2$, $\text{R}_1 = \text{R}_2 = \text{CH}_2\text{CH}_2\text{OH}$
- (4) $n = 2$, $\text{R}_1 = \text{CH}_2\text{CH}_2\text{OH}$, $\text{R}_2 = \text{Ph}$

Fig. 2. 1 Structure of alkylamino side chains

Two synthetic pathways were explored in the preparation of the various hydroxyethylamino side chains. In the first (Kohn *et al.*, 1994), bromoalkanonitrile (alkano = $(\text{CH}_2)_n$; $n = 2$ or 3) was reacted with the secondary amine 2-(methylamino)ethanol to give the (*N*-methyl-*N*-hydroxyethyl)alkanonitrile. The nitrile group was then reduced to a primary amine with lithium aluminium hydride and the crude product distilled under high vacuum to give side chains (1) or (2). A different synthetic strategy was applied for the synthesis of sidechains (3) and (4). In the first method, ethylenediamine was monoprotected with ethylacetate (Aspinall, 1949) and the free primary amine reacted with excess ethylene oxide to give the acetyl protected *N,N*-bis-(hydroxyethyl)ethylenediamine (Peck *et al.*, 1959). Removal of the protecting group with dilute acid followed by high vacuum distillation afforded the product (3) as an oil. In the synthesis of (4), the primary amine of 2-(*N*-phenyl)ethylenediamine was protected with diphenylacetophenone. Alkylation at the secondary amine with ethylene oxide and deprotection to isolate the product followed similar reaction conditions to the synthesis of (3) (Peck *et al.*, 1959 and 1961). All sidechains were isolated with 60-80% purity and identified by H^1 NMR which corresponded with previous literature values.

The substitution of the sidechains onto the anthraquinone chromophore to give the mono (Fig. 2. 2), and the 1,4 disubstituted compounds as the 5,8-dihydro- and 5,8-dihydroxyanthraquinones (Fig. 2. 3) followed the general synthetic procedure of Murdock *et al.*, (1979) and Johnson *et al.*, (1979). Synthesis of the 5,8 dihydro- and 5,8 dihydroxyanthraquinones took place under a nitrogen atmosphere following the addition of the appropriate amine to leucoquinizarin or 5,8-dihydroxyleucoquinizarin, respectively. Monitoring the reaction by TLC showed the initial formation of a purple spot, the mono substituted product (approximate R_F 0.75) and this was followed by the formation of a blue spot, the product (approximate R_F 0.25). Air oxidation overnight, followed by column chromatography and recrystallisation gave the product as a blue powder. Mono substituted anthraquinones were isolated as a red/orange powder following recrystallization.

The synthesis of the mixed sidechain compounds (Fig 2. 4) was achieved according to the literature method of Krapcho *et al.*, (1990) and Stefanska *et al.*, (1989). Two side chains were required for this reaction and TLC showed the formation of three blue spots of approximate equal intensity, but with differing polarities. Isolation of the middle spot by column chromatography and recrystallisation gave the product as a blue powder.

Conversion of the hydroxyethylaminoanthraquinones to chloroethylaminoanthraquinones required the reaction to proceed at room temperature in triphenylphosphine/carbontetrachloride with dichloromethane as solvent. This method was adapted from Slagle *et al.*, (1981). Following recrystallization, the chloroethylaminoanthraquinones were isolated in approximately 80% yield. By TLC the product was observed as a single spot with non-polar properties (R_F 0.8). In some cases a difficulty was encountered when dissolving the starting material and therefore acetonitrile was added to the reaction to increase solubility. All compounds were identified by 1H NMR, FAB+ MS, UV/VIS and IR.

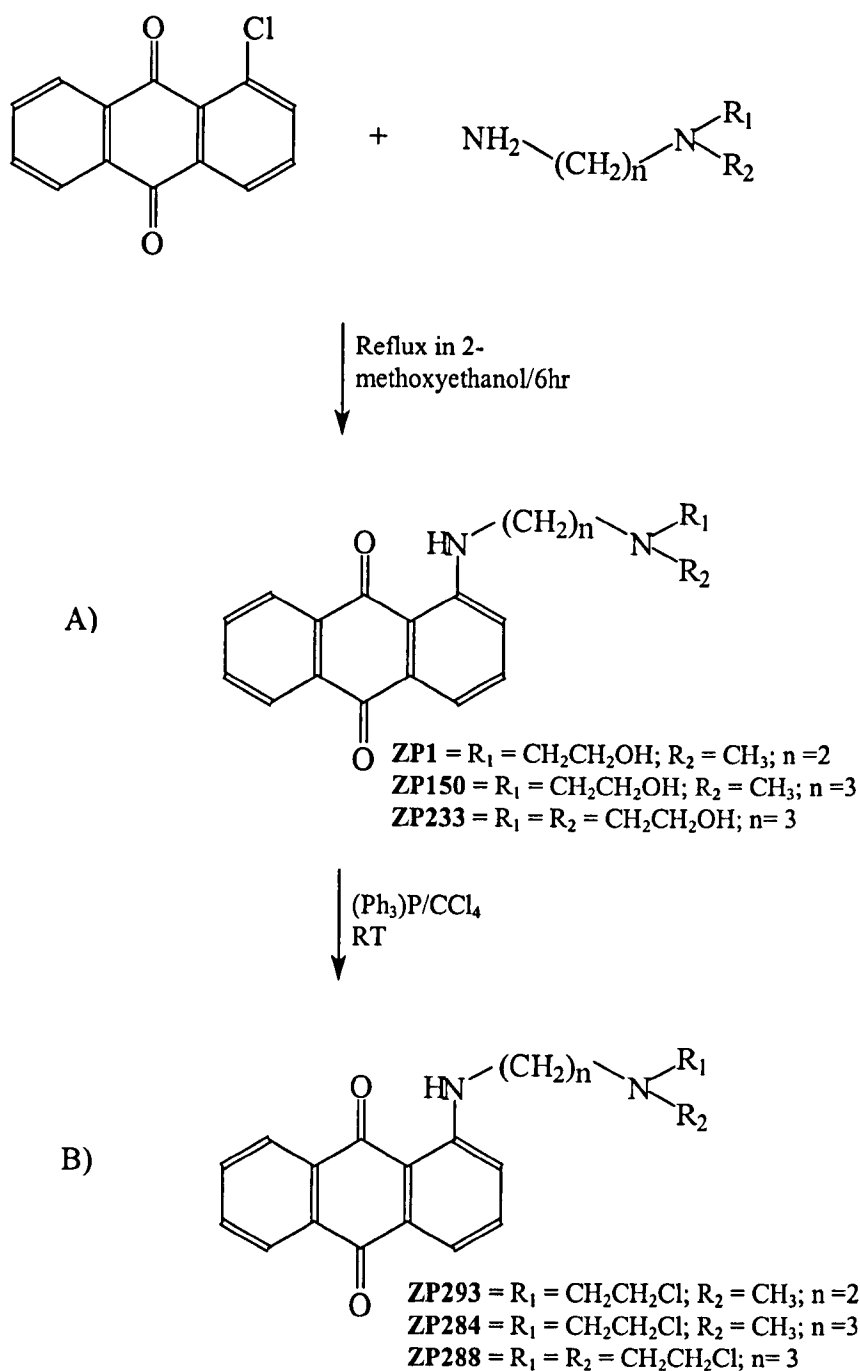
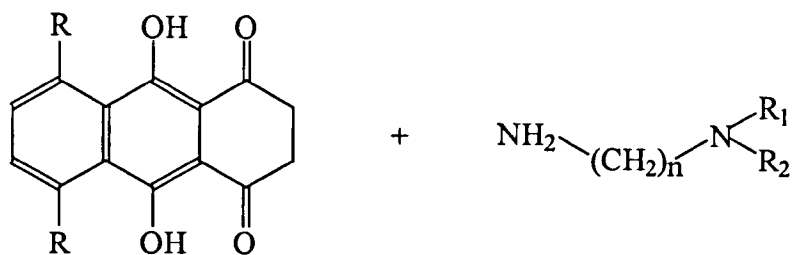


Fig. 2. 2 General reaction scheme for the formation of mono anthraquinones
 a) substitution to give the hydroxyethylaminoanthraquinone
 b) halogenation to give the chloroethylaminoanthraquinone.



**5,8-dihydroxy-
hydroxyethylaminoanthraquinones:**

R = OH

ZP254 = R₁ = CH₂CH₂OH; R₂ = Ph; n = 2

ZP255 = R₁ = R₂ = CH₂CH₂OH; n = 3

ZP265 = R₁ = CH₂CH₂OH; R₂ = CH₃; n = 2

ZP273 = R₁ = CH₂CH₂OH; R₂ = CH₃; n = 3

heat for
5hr

**5,8-Dihydro-
hydroxyethylaminoanthraquinones:**

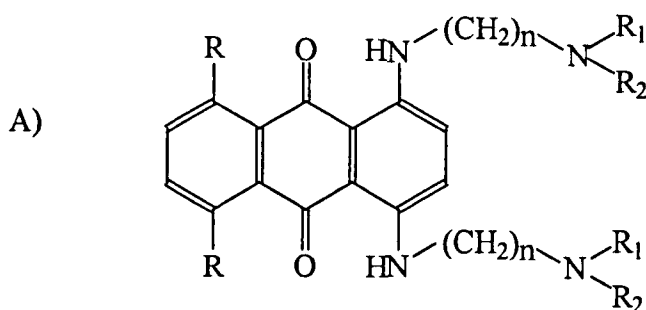
R = H

ZP232 = R₁ = R₂ = CH₂CH₂OH; n = 2

ZP240 = R₁ = R₂ = CH₂CH₂OH; n = 3

ZP242 = R₁ = CH₂CH₂OH; R₂ = CH₃; n = 2

ZP245 = R₁ = CH₂CH₂OH; R₂ = Ph; n = 2



**5,8-Dihydroxy-
chloroethylaminoanthraquinones:**

R = OH

ZP280 = R₁ = R₂ = CH₂CH₂Cl; n = 2

ZP282 = R₁ = R₂ = CH₂CH₂Cl; n = 3

ZP285 = R₁ = CH₂CH₂Cl; R₂ = CH₃;
n = 2

(Ph₃)P/CCl₄
RT

**5,8-Dihydro-
chloroethylaminoanthraquinones:**

R = H

ZP286 = R₁ = CH₂CH₂Cl; R₂ = CH₃;
n = 2

ZP289 = R₁ = R₂ = CH₂CH₂Cl; n = 3

ZP290 = R₁ = R₂ = CH₂CH₂Cl; n = 2

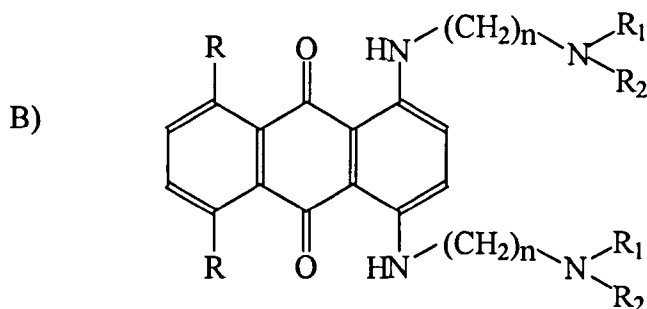


Fig. 2.3 General reaction scheme for the formation of 1,4 disubstituted anthraquinones a) hydroxyethylaminoanthraquinones
b) chloroethylaminoanthraquinones

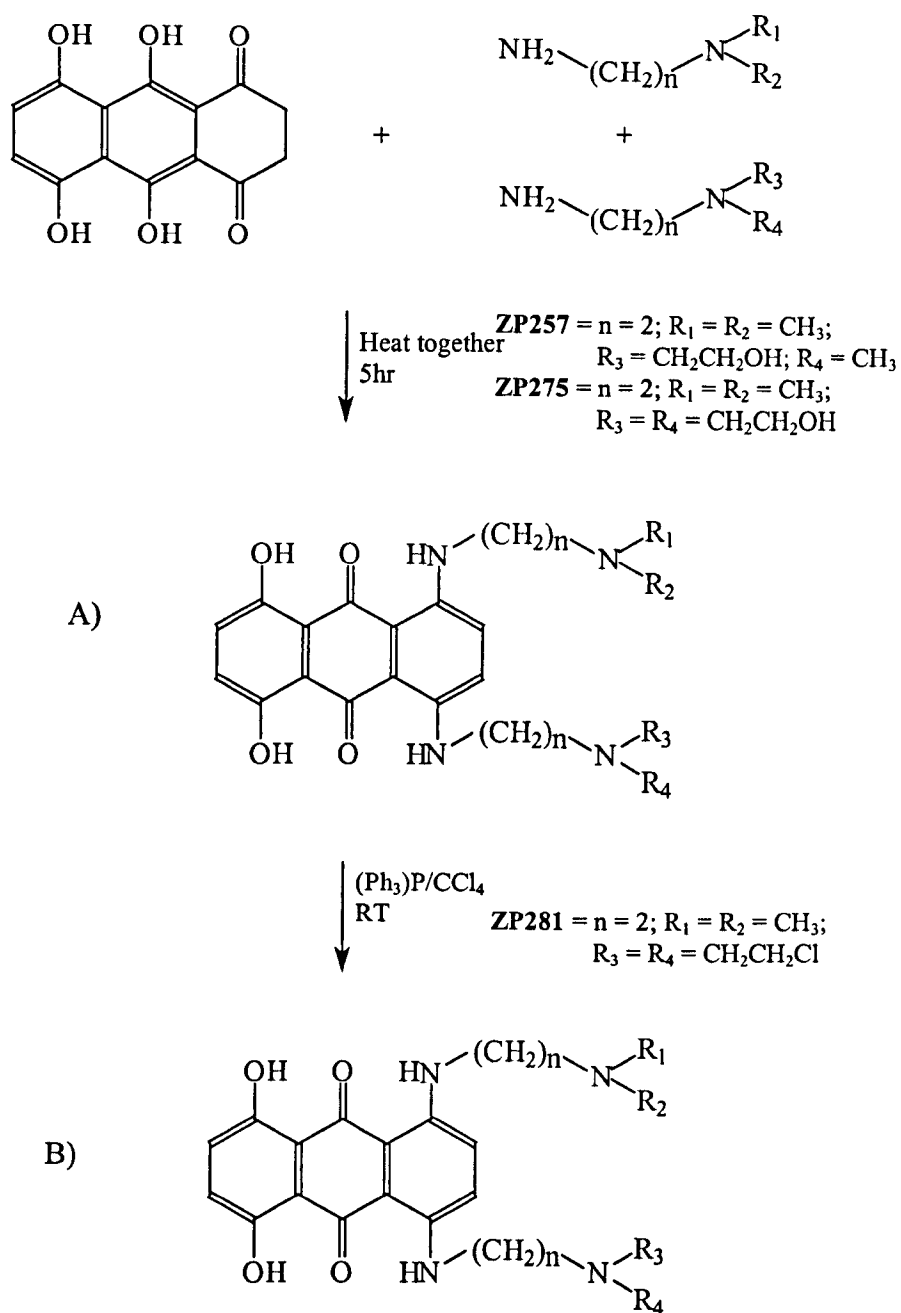


Fig. 2. 4 Reaction scheme for the formation of unsymmetrical 1,4 disubstituted anthraquinones to give
 a) hydroxyethylaminoanthraquinones and
 b) chloroethylaminoanthraquinones

2. 6 DISCUSSION

2. 6. 1. SYNTHESIS OF HYDROXYETHYLALKYLAMINO SIDE CHAINS

The synthesis of side chains (1) and (2) was adapted from the literature methods of Peck *et al.*, (1959 and 1961) and Kohn *et al.*,(1994). Following S_N2 substitution of the secondary amine onto a bromoalkanonitrile, the nitrile group was easily converted by reduction with lithium aluminium hydride to a primary amine. Because of the viscous nature and high boiling point of the product, difficulties were encountered during purification and therefore a second procedure was explored.

The use of ethyl acetate to protect primary amines has previously been described by Aspinall, (1941). The ready availability of this common laboratory solvent meant that the protection procedure could be performed on a large scale. Ethylenediamine was stirred with ethyl acetate at room temperature over a seven day period (Fig 2. 5) and the mono-protected product distilled to remove impurities such as the disubstituted amine. The free primary amine was then dialkylated with an excess of ethylene oxide (Peck *et al.*,1959) to give the N, N'-bis-(hydroxyethyl)tertiary amine (3) (Fig . 2 . 6)

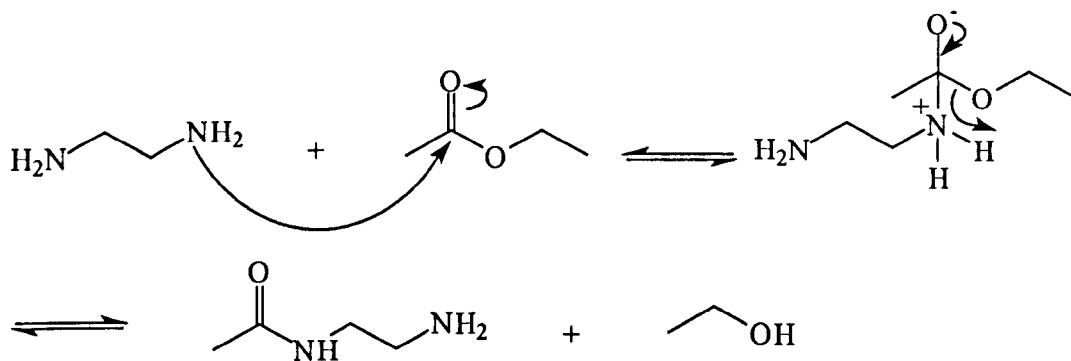


Fig. 2. 5 Protection of primary amine with ethyl acetate

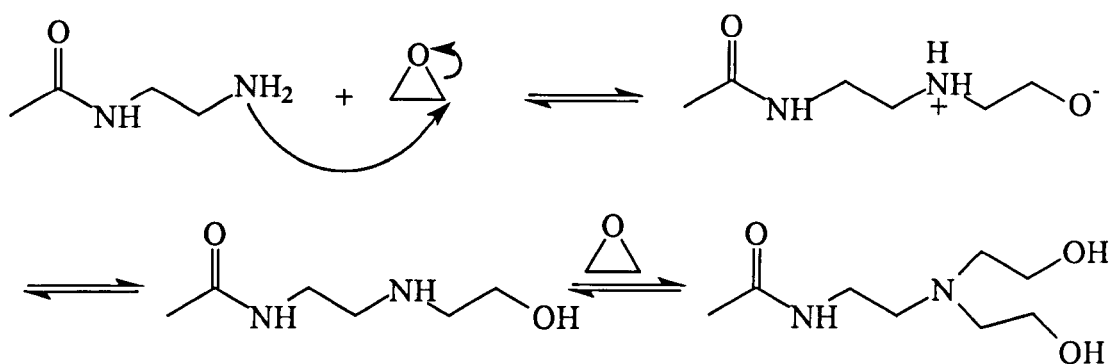


Fig. 2. 6 Reaction of primary amine with ethylene oxide to form hydroxyethyl side arm.

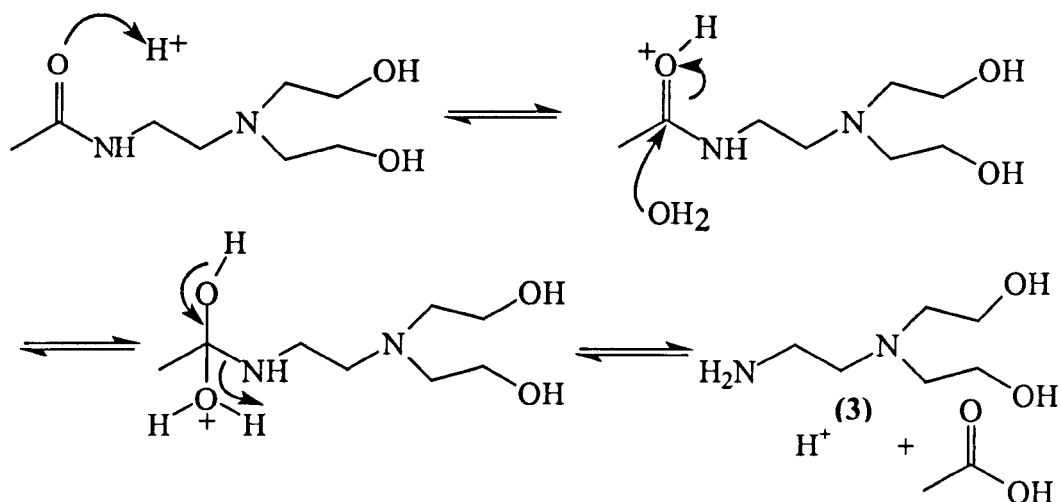


Fig. 2. 7 Deprotection of the primary amine under acid conditions

Although this synthetic route was successful, the length of time required for the first step of the reaction to reach completion led to the search for an alternative protecting group for the synthesis of the *N*-2-(Hydroxyethyl)-*N*-phenylethylenediamine side chain (4). When considering ketones as protecting agents, it is necessary for the carbonyl group to be substituted with at least one aryl group so as to stabilise the protected product to decomposition (March, 1992). The ketone benzophenone was found to be appropriate as it

is selective for the primary amino function and it can easily be displaced when required. As *N*-Phenyl-ethylenediamine was the starting material for the reaction, the formation of the di-protected amine was precluded. Primary amines react with ketones via nucleophilic addition to the carbonyl group to form a tetrahedral intermediate (Fig. 2. 8) and this is followed by the loss of water to form an imine as the protecting group. In order to increase the rate of reaction, a catalyst, borontrifluoride etherate was employed and the water formed as a byproduct was removed by azeotropic distillation to drive the reaction to completion.

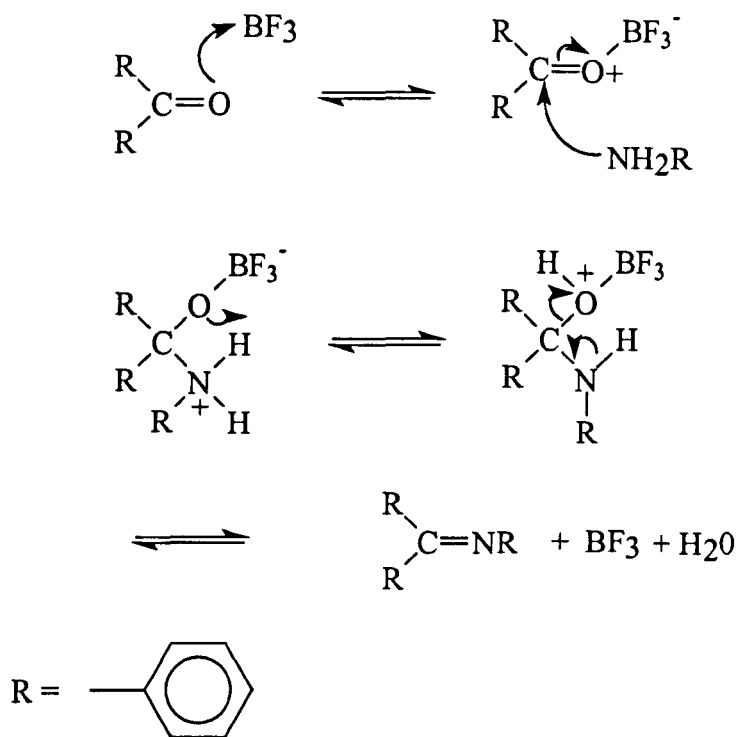


Fig 2. 8 Protection of primary amine with benzophenone

2. 6. 2. CHROMOPHORE SUBSTITUTION REACTIONS

Much of the synthesis of monosubstituted and 1,4-disubstituted anthraquinones revolves around the chemistry of aromatic nucleophilic substitution. Generally, compounds such as the halobenzenes are resistant to this type of reaction due to the electron rich nature of the aryl system. However, when an activating group such as a carbonyl or nitro group is

located ortho and/or para to the halogen, substitution can be rationalised by the S_NAr mechanism (March, 1992) which takes place in two steps. The attacking species forms a bond with the substrate producing an intermediate complex and then the leaving group departs. The intermediates formed are similar to Meisenheimer salts (Fig. 2. 9). By comparison, the chemistry of mono-substitution onto the anthraquinone moiety is dominated by the electron withdrawing property of the two quinone carbonyl groups which generate a partial charge across the three rings and weaken the carbon-halogen bond, activating it to nucleophilic substitution.

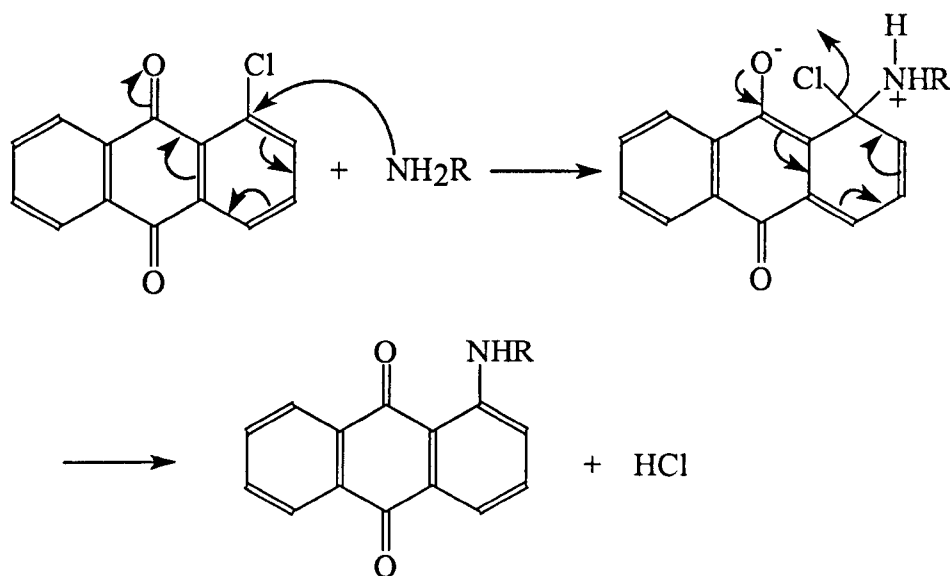


Fig. 2. 9 Formation of mono anthraquinones

The synthesis of ZP1, ZP233 and ZP150 was achieved by treating 1-chloroanthraquinone with a ten fold excess of amine in the solvent 2-methoxyethanol, and the mixture refluxed for 6 hours. At first attempt this reaction was performed by heating the two reactants together as the amine, a viscous liquid, was thought suitable to act as both nucleophile and solvent. Despite a long reflux time, 48 hours, no improvement in yield above 10% could be achieved. The poor reactivity was initially attributed to the lack of absolute purity of the side-chains and the possibility of a more reactive nucleophile

substituting onto the chromophore, however this was not evident by TLC. Instead, visualisation under UV light revealed that a large amount of unreacted 1-chloroanthraquinone was present in the reaction mixture indicating that the solubility of the starting material was poor. In view of this, a solvent, 2-methoxyethanol was selected to dissolve 1-chloroanthraquinone at elevated temperatures (129°C) and allowed the reaction to be controlled at reflux. Only a 5% increase in yield was attained by using this solvent and so further modifications to the method were made.

When various alkylamino side chains were reacted with 9-chloro-3-methoxyacridine, Kohn *et al.*, (1994) found that the hydrogen chloride displaced as a by-product of the reaction was effectively protonating the primary amine side chain and preventing it from substituting onto the chromophore. Consequently, the chlorine atom of acridine was replaced with a phenoxy group which allowed couplings to proceed without the need for an excess of amine. This procedure was followed for the 1-chloroanthraquinone, however poor reactivity with the phenol prevented this route from being further pursued. Instead, pyridine was added to complex the hydrogen chloride expelled. The outcome was to increase the overall yield by only 2% which suggests that protonation of the amine side chain was not a major concern regarding this reaction. As the final yield of 17% seemed the best available and provided adequate product for subsequent steps, no further work was done on this synthesis.

The preparation of 5,8-dihydro and 5,8-dihydroxyanthraquinones has been described extensively in the literature (Zee-Cheng *et al.*, 1978; Murdock *et al.*, 1979; Johnson *et al.*, 1979). Generally the reaction mechanism involves the condensation of leucoquinizarins (Fig. 2. 3 and 2. 4) with an excess amount of the appropriate amine. The initial reaction of the primary amine is at the quinone group on the 1-position of the chromophore followed by the loss of water which results in the formation of an imine. This is followed by a second identical slow reaction at the 4-position. The intermediate imines (Schiff bases) are then oxidized in air or by the addition of a few drops of base to secondary aryl amines via internal rearrangement of the anthraquinone chromophore. The oxidation step can be

readily monitored by TLC as a colour change from orange/brown to blue is observed on conversion to products.

When mitoxantrone is synthesised, reaction temperatures are maintained below 50°C as the N-monosubstituted ethylenediamine side chain can readily cyclise onto the 2-position of the anthraquinone ring to form the hydroxyethylamino-1,2,3,4-tetrahydronaphtho-(2,3-f)-quinoxaline-7,12-dione (see Fig. 1. 6) (Murdock *et al.*, 1979). In contrast, the tertiary nature of the hydroxyethylaminoanthraquinone side chains prevents a similar intramolecular rearrangement from taking place.

It is generally accepted that the reaction of an amine with leucoquinizarin generates a low quantity of product. As a consequence this has led to the pursuit for a more reactive anthraquinone chromophore substituted with functional groups that can be displaced more readily by the amine nucleophile. Krapcho *et al.*, (1990) devised two methods to synthesise 1,4-difluoro-5,8-dihydroxy-9,10-anthracenedione which on further reaction gave mitoxantrone with a 78% yield. More recently Lee *et al.*, (1999) described a five step synthesis to isolate AQ4 from the starting product 3, 6 dichlorophthalic anhydride. Whilst being high yielding, the expense of these routes and the additional steps required in the preparation of the starting material make them relatively unattractive.

2. 6. 3 SYNTHESIS OF CHLOROETHYLAMINOANTHRAQUINONES

In the conversion of an alcohol to an alkyl halide, the reaction can be initiated either at the -C-O- bond or at the -O-H bond. As the hydroxyl is a poor leaving group it does not undergo S_N2 reactions readily. To overcome this, much use is made of inorganic halides such as SOCl₂, PCl₅, PCl₃ and POCl₃ which form inorganic ester intermediates that can easily be displaced by a halide ion (Fig. 2. 10 and see March, 1992 for references).

The reaction of an alcohol with thionyl chloride (acting as both solvent and nucleophile) follows a second order rate equation ($\text{Rate} = k[\text{ROH}][\text{SOCl}_2]$) with retention of configuration and proceeds via an S_Ni (internal nucleophilic substitution) mechanism (Fig 2. 10). The rate at which the alkylchlorosulphite intermediate breaks down to the product RCl is found to increase with increasing polarity of the solvent, and also with

increasing stability of the carbocation R^+ , therefore, an ion pair, R^+OSOCl^- is thought to be involved. Following collapse of the ion pair to products, attack is likely to occur on the same side of R^+ from which ^-OSOCl departed and as a result configuration is retained. If the reaction is carried out in the presence of pyridine, the product undergoes inversion of

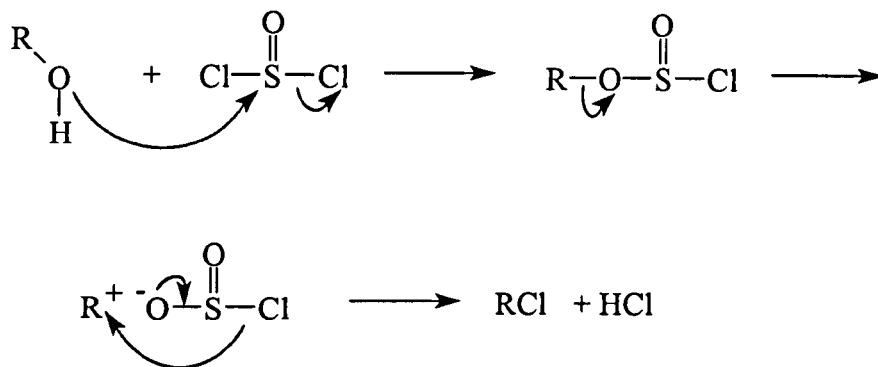


Fig. 2. 10 S_Ni reaction of thionyl chloride with a hydroxyl group

configuration. The HCl produced during the reaction complexes with the pyridine and Cl^- then attacks from the back as expected for an S_N2 reaction.

Inorganic halides such as phosphorus oxychloride and thionyl chloride have been successfully employed in the synthesis of substituted acridine anti-tumour drugs (Peck *et al.*, 1961; Kohn *et al.*, 1994, Watanabe *et al.*, 1989) and quinoline anti-malarial drugs (Peck *et al.* 1959), with thionyl chloride being the more favoured reagent. Following the method of Kohn *et al.*, (1994), a 100 fold excess of thionyl chloride was reacted with the hydroxyethylaminoanthraquinone in the absence of air to give a mixture from which the product could not be isolated pure by recrystallisation. Purification by column chromatography proved highly problematical as the product could not be separated from the top of the silica column. In an effort to reduce impurities, attempted modifications to the procedure such as increasing the temperature of reaction to 40 °C and the use of additional solvents such as dimethylformamide (Watanabe *et al.*, 1989) and diethylether were investigated without success. An increase in the quantity of product formed was observed

when the starting material ZP1 was protonated with ethereal hydrogen chloride prior to halogenation. Despite this, the impurities present could not be eliminated completely (see Appendix 3 for HPLC data) and therefore an alternative method for halogenation was explored.

Triphenyl phosphine has for a long time been used in conjunction with carbon tetrachloride in the halogenation of alcohols (reviewed by Castro, 1983) such as in the synthesis of the chlorambucil analogue [3-(trifluoromethyl)chlorambucil] (Coe *et al.*, 1997) which was achieved in good yield. The reaction proceeds in a two step mechanism which involves the initial formation of a cluster of ion pair intermediates stabilized by the positively charged phosphorous in one ion pair adjacent to a negative chloride from another ion pair and this is followed by intermediate decomposition (see Fig 2. 11) (Slagle *et al.*, 1981). It has been shown that decomposition proceeds unimolecularly with inversion of configuration and that the rate of formation of products is only slightly influenced by steric effects (Jones *et al.*, 1978). When a solvent such as acetonitrile is used during the reaction halogenation is

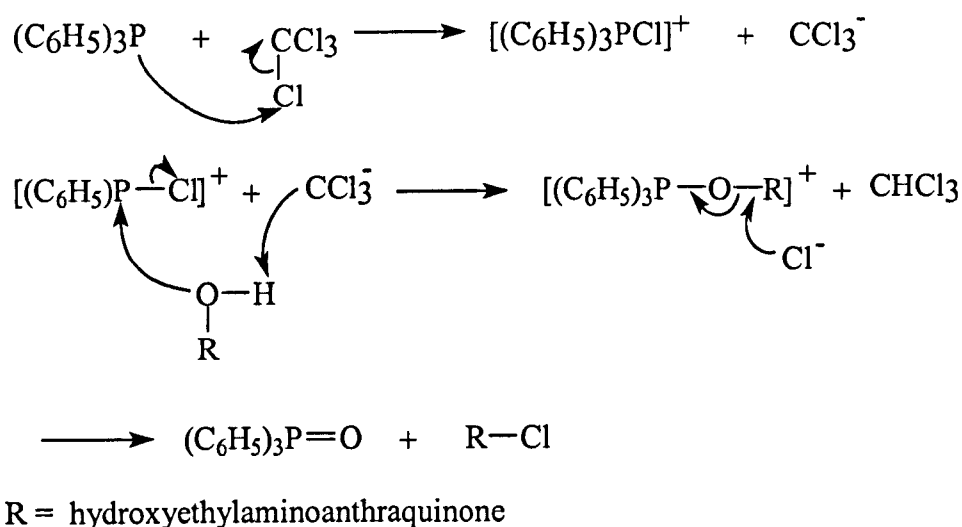


Fig.2. 11 Synthetic route for the conversion of hydroxyethylamino-anthraquinones to chloroethylaminoanthraquinones

reported to proceed very rapidly and the enhanced polarity is effective enough to change the kinetic order from unimolecular to bimolecular (Slagle *et al.*, 1981). This method of chlorination proved to be the most effective, resulting in 70-85% yield following recrystallisation for all compounds isolated. Only one compound ZP292 proved difficult to halogenate because of its poor solubility in the solvent dichloromethane. The NMR of this compound demonstrates that one side chain underwent chlorination to give the half mustard whilst the remaining material was unreacted ZP254 (starting material).

Fig. 2. 11 describes the formation of the ion pair intermediate through nucleophilic attack onto a chlorine atom of carbon tetrachloride. In an opposing view, Castro *et al.*, (1983) suggests that this step could only take place if a highly nucleophilic reagent such as aminophosphine replaced triphenylphosphine during reaction, as a strong nucleophile would allow complete charge transfer to the trichloromethyl anion to occur. Instead, in an amendment to the mechanism described in Fig. 2. 11, it was postulated that triphenylphosphine attacks the tetrachloromethane carbon and forms an ion pair with the displaced chloride anion.

2. 6. 4 ATTEMPTED SYNTHESIS OF CHLOROETHYLAMINOANTHRAQUINONE N-OXIDE HYDROCHLORIDE

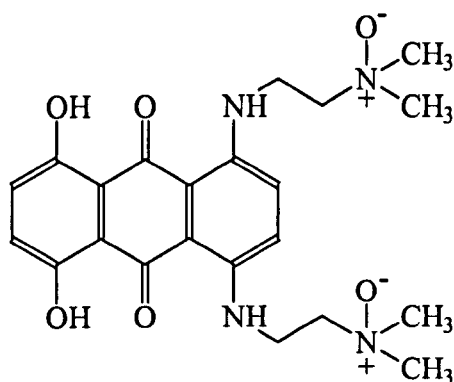


Fig. 2. 12 AQ4N

The drug AQ4N (Fig. 2. 12) was developed as a hypoxia selective bis-bioreductive prodrug which undergoes cellular reduction at the N-oxide to give a DNA binding agent that is cytotoxic through the inhibition of topo II (Patterson, 1989, 1993). The inclusion of a neutral quarternary N-oxide salt in the drug structure considerably decreases the attraction of AQ4 for DNA and coincides with minimal prodrug cytotoxicity (Patterson, 1993). It was therefore anticipated that a similar but much improved spectrum of activity would be attained if a nitrogen mustard N-oxide could be linked to an anthraquinone chromophore. Previous reports of aryl mustard N-oxide synthesis demonstrated that the compounds generated were unstable. In acidic medium they reform to the original nitrogen mustard by loss of the tertiary amine oxygen, and in basic media they rearrange to the half mustard and a simple aldehyde (Degutis *et al.*, 1972; Degutene *et al.*, 1986, Tercel *et al.*, 1995). Regarding this, an attempt was made to synthesize an N-oxide of the anticancer drug chlorambucil which was initially considered to be successful (Kirkpatrick *et al.*, 1994). However, an investigation into its decomposition revealed that a stable hydroxylamine was formed as the major rearranged product in organic solvents and this had been isolated instead (Tercel *et al.*, 1995).

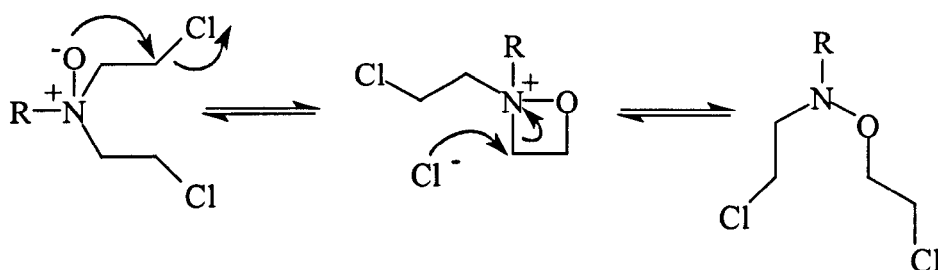


Fig. 2. 13 Proposed rearrangement of aliphatic bis-mustard N-oxides via a cyclic intermediate

Despite the futile attempts to isolate an aryl mustard N-oxide, an aliphatic bifunctional mustard N-oxide of mechlorethamine (Nitromin) had been synthesised previously. Although aliphatic bifunctional mustard N-oxides are known to undergo fast rearrangement via a cyclic intermediate to give trisubstituted hydroxylamines (Fig. 2. 13),

nitromin has demonstrated increased stability and reduced cytotoxicity (30-40 fold) over mechlorethamine with some selectivity for hypoxic cells (Connors, 1988; White *et al.*, 1989). Furthermore, an investigation into the decomposition of monofunctional mustard N-oxides in aqueous media revealed that they hydrolyse more slowly than their bifunctional equivalents (Owari *et al.*, 1953). Based on this evidence it was considered possible to prepare a stable aliphatic chloroethylaminoanthraquinone N-oxide. The proposed starting point was the isolation of a simple mono functional N-oxide by reacting mono substituted chloroethylaminoanthraquinone ZP293 with mCPBA to form ZP180 (see Fig 2.14).

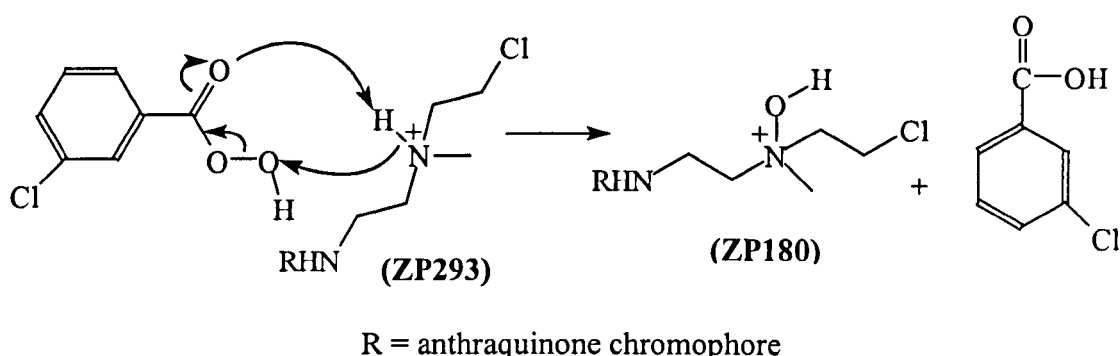


Fig. 2.14 Attempted synthetic route for the formation of a mustard N-oxide

This reaction followed a modified method of Patterson *et al.*, (1989). The N-oxide was isolated pure (observed by TLC) following column chromatography but decomposed on solvent evaporation. Subsequent FAB+MS analysis showed that the mixture contained a molecular ion $[M]^+$ peak consistent with the relative molecular mass of the mustard N-oxide. However, it is unknown whether this was the desired product or the product of rearrangement to the hydroxylamine, as both compounds would give identical MS spectra. Because rearrangement of even the monofunctional anthraquinone mustard N-oxide proved facile, no further attempts at N-oxidation were made.

To summarise, a number of hydroxyethylaminoalkylamino sidechains were synthesised and substituted onto an anthraquinone chromophore to give a series of mono substituted and 1, 4-disubstituted hydroxyethylaminoanthraquinones. The subsequent

conversion of the hydroxyethylaminoanthraquinones to chloroethylaminoanthraquinones involved initial attempts at halogenation using thionyl chloride. However, the large number of impurities produced from reaction and a difficulty in purification led to the choice of milder chlorinating reagent triphenylphosphine with carbontetrachloride. This method proved to be high yielding with few impurities and was therefore utilised to produce a series of derivatives that were put forward for further study. An attempt was also made to isolate a simple mono mustard anthraquinone N-oxide which, due its instability, led to the abandonment of further synthesis.

AN INVESTIGATION INTO THE MECHANISM
OF ACTION OF ALKYLATING
ANTHRAQUINONES

Chapter 3 DNA-BINDING STUDIES

3.1. INTRODUCTION

DNA intercalating ligands have the capacity to bind reversibly to DNA by insertion of an aromatic chromophore between DNA base pairs. One aspect of intercalative binding that influences anti-tumour activity is the drug binding strength with DNA (reviewed by Denny *et al.*, 1989). This principle is not always followed, as there are many intercalating agents that bind tightly to DNA but have no activity (Feigon *et al.*, 1984). Despite this, drug/DNA binding which can be measured in terms of drug/DNA association constant (K_1), drug dissociation rate (k_d) from DNA (Gandecha *et al.*, 1985; Denny *et al.*, 1989) or more precisely the average residence time spent by the chromophore at a particular binding site (Denny *et al.*, 1990), has often demonstrated a positive correlation with cytotoxicity within a given class of compound (Denny *et al.*, 1989).

A series of novel chloroethylaminoanthraquinones and hydroxyethylaminoanthraquinones were compared for their ability to intercalate into calf thymus DNA, and their DNA association constants (K_1) and frequency of base pair intercalation (n) determined. Furthermore, the effect of drug stabilisation of the DNA helix to thermal denaturation (T_m) was investigated in order to distinguish between covalent and non-covalent drug-DNA interactions.

3.2. CHEMICALS AND REAGENTS

Calf thymus DNA was purchased from Sigma Chemicals, London, UK. Tris, hydrochloric acid and sodium chloride were purchased from Fisher Scientific, Loughborough, UK. All drug samples were stored as a dry powder and dissolved into DMSO to give a 50 μ M stock solution prior to being further diluted to the specified concentration in the appropriate buffer.

3.3 METHODS

For preparation of DNA solution and high and low ionic strength buffers see appendix 1 and 2 respectively.

3.3.1. DETERMINATION OF DRUG/DNA ISOSBESTIC POINT

A series of DNA/Drug ratios (0, 0.2, 0.5, 1, 2, 5, 10, 15 to 1) were prepared by varying the concentration of DNA from 0 to 2.25×10^{-4} M against a constant concentration of drug 1.5×10^{-5} M. To eight 10ml volumetric flasks was added drug (1.5×10^{-5} M) and high ionic strength buffer to give a final volume of 9ml. The appropriate ratio of DNA was then carefully added to each flask and the final solution accurately made up to 10ml with buffer and mixed by single inversion. The spectra of each concentration ratio was recorded in the visible region (350-700nm), and superimposed to give eight overlapping spectra. This method was repeated using low ionic strength buffer.

3.3.2. DETERMINATION OF THE BINDING CONSTANT OF DRUG WITH DNA

A stock solution of DNA (1×10^{-3} M) in high ionic strength buffer was prepared according to appendix 1. The drug (2.5×10^{-5} M) in buffer (3ml) was added to a 1cm pathlength quartz cuvette and its absorbance measured to determine the drug λ_{\max} . DNA stock solution was serially pipetted into the drug solution at volumes of: $12 \times 10 \mu\text{l}$, $6 \times 20 \mu\text{l}$, $8 \times 40 \mu\text{l}$ and $4 \times 100 \mu\text{l}$. After each addition the solution was stirred and left to equilibrate for 10 minutes after which, the absorbance at the λ_{\max} of the drug was taken. All data were reproduced in triplicate.

3.3.3 DETERMINATION OF THE THERMAL DENATURATION OF DNA

Low ionic strength buffer (3ml) and distilled (5.5ml) was added to a 10 ml volumetric flask and degassed by sonication for 30 min. A solution of DNA (3.2×10^{-6} M) in low ionic strength buffer (0.6ml) was slowly added to the flask and the remaining volume made up to 10 ml with dH_2O . The solution was mixed by a single inversion of the flask and pipetted into two 1cm pathlength quartz cells, the reference and sample cells. The

temperature of the reference cell was maintained at room temperature whilst the sample cell temperature was raised to 50°C and the solution left to equilibrate for 15 min. The absorbance of the DNA was monitored at 260nm as the sample cell temperature was raised at a rate of 1°C/min and readings taken at 1°C intervals from 50°C-85°C. All data were reproduced in triplicate.

3. 3. 4 DETERMINATION OF THE THERMAL DENATURATION OF DNA IN THE PRESENCE OF HYDROXYETHYLAMINOANTHRAQUINONE

Low ionic strength buffer (0.75ml), dH₂O (6ml) and drug (8.5×10^{-8} M) was added to a 10ml volumetric flask and degassed by sonication for 30min. DNA (8.5×10^{-7} M) in low ionic strength buffer (0.6ml) was slowly added to the flask and the volume made up accurately to 10ml with dH₂O to give a final DNA:drug ratio of 10:1. The solution was mixed by single inversion of the flask and pipetted carefully into two 1cm pathlength quartz cells, the reference and sample cells. The temperature of the reference cell was maintained at room temperature whilst the sample cell temperature was raised to 60°C and the solution left to equilibrate for 15 minutes. The absorbance of the DNA was monitored at 260nm as the sample cell temperature was raised at a rate of 1°C/min and readings taken at 1°C intervals from 60-100°C. All data were reproduced in triplicate.

3. 3. 5. DETERMINATION OF THE THERMAL DENATURATION OF DNA IN THE PRESENCE OF CHLOROETHYLAMINOANTHRAQUINONE

Low ionic strength buffer (0.75ml) and dH₂O water (6ml) were added to a 10ml volumetric flask and degassed by sonication for 30min. To this was added the drug (8.5×10^{-8} M) and then the DNA (8.5×10^{-7} M) and the volume made up accurately to 10ml with dH₂O water. The solution was mixed by single inversion of the flask and pipetted into two 1cm pathlength quartz cells, the reference and sample cells. The temperature of the reference cell was maintained at room temperature whilst the sample cell temperature was raised to 60°C and the solution left to equilibrate for 15 minutes. The absorbance of the

DNA was monitored at 260nm as the sample cell temperature was raised at a rate of 1°C/min and readings were taken at 1°C intervals from 60-100°C.

All data were reproduced in triplicate.

3. 4. RESULTS

3. 4. 1 DETERMINATION OF ISOSBESTIC POINT

DNA intercalating agents such as the anthraquinones absorb in the visible region of the electromagnetic spectrum to give characteristic absorption spectra. If the spectra derived from the addition of varying concentrations of DNA to a single concentration of drug is superimposed onto the spectrum of the drug alone, the absorbance wavelength at which all the spectra cross is termed the isosbestic point. This may be achieved if there are only two species in solution, that is, free drug and DNA bound drug. The point at which DNA is fully bound with drug corresponds to no further decrease in the extinction coefficient at the λ_{max} of the free drug.

The isosbestic behaviour of eight hydroxyethylaminoanthraquinones was determined in high ionic strength buffer (0.5M NaCl). Unlike the 1, 4-disubstituted anthraquinones (Fig. 3.1 and Fig. 3. 2) the mono derivatives showed no apparent affinity for calf thymus DNA. The 5, 8-dihydroanthraquinones could be distinguished from the 5, 8-dihydroxy derivatives by the position of their isosbestic point, 639 nm and 618 nm respectively (Table 3. 1). Furthermore, the spectral shifts for the 5, 8-dihydroxyanthraquinones were up to 50% greater than for the 5,8-dihydroanthraquinones indicating enhanced chromophore π - orbital interaction with the DNA base pairs.

In contrast to the other compounds tested ZP274, a 5,8-dihydroxyanthraquinone substituted with two hydroxyethyl groups per side chain gave an isosbestic point at the four lowest drug concentrations in low ionic strength buffer (0.05M) (Fig. 3. 3). This behaviour has been observed previously (Kapuszcinski *et al.*, 1985) and may be related to the

preference of this compound at high drug/DNA concentration to bind electrostatically to the surface of DNA.

The isosbestic behaviour of ZP254 was not determined as drug precipitation occurred on addition to buffer. The bulky phenyl groups attached to the side chains of ZP254 increase its lipophilic character and therefore make it poorly soluble in water even if the concentration of DMSO in buffer is raised to 10%.

The spectra of ZP285 (Fig. 3. 4) is representative of the effect of the chloroethylaminoanthraquinones on DNA binding. At each increasing DNA concentration the drug absorbance showed no change in λ_{max} but demonstrated a large change in its visible region when compared to the 1,4-disubstituted-5,8-dihydroxy-hydroxyethylaminoanthraquinones (Fig. 3. 2). When the same concentration of drug was added to distilled water in the absence of salt and buffer, the characteristic 1, 4-disubstituted anthraquinone spectra was re-gained (Fig. 3. 5). This indicates that high ionic strength buffer may adversely affect the intercalation of the chloroethylaminoanthraquinones and reduce their affinity for DNA.

Table 3.1 The effect on visible absorbance following the addition of the hydroxyethyanthraquinones to calf thymus DNA.

Compound	I. P (nm)	Shift in λ_{max} (nm)
ZP232	639	10
ZP240	639	20
ZP242	636	17
ZP255	618	20
ZP257	618	18
ZP265	618	20
ZP274*	618	n/d
ZP275	618	17

I.P = Isosbestic point (nm); shift in λ_{max} (nm) from free drug to fully intercalated DNA. n/d= not determined, * isosbestic point determined in low ionic strength buffer (0.05M).

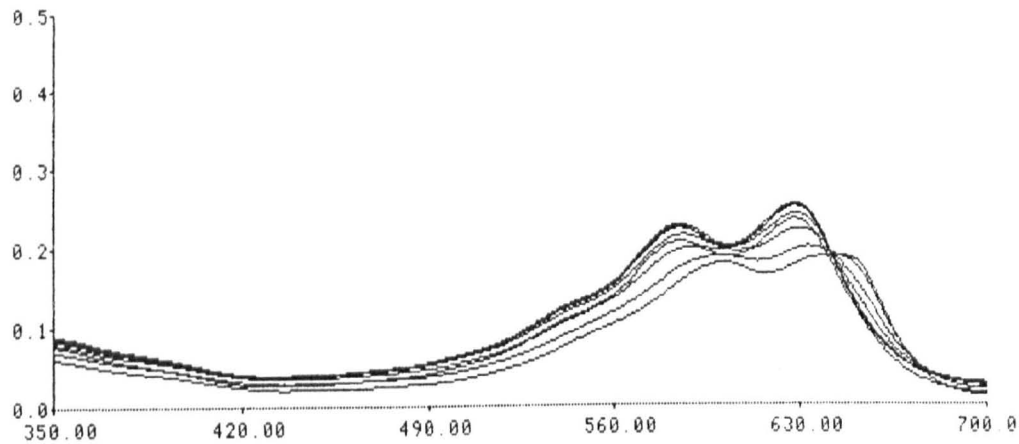


Fig 3. 1 Spectral shift in visible absorbance following the addition of increasing concentrations of calf thymus DNA to 5,8- dihydro- hydroxyethylaminoanthraquinone ZP242. Determined in high ionic strength buffer.

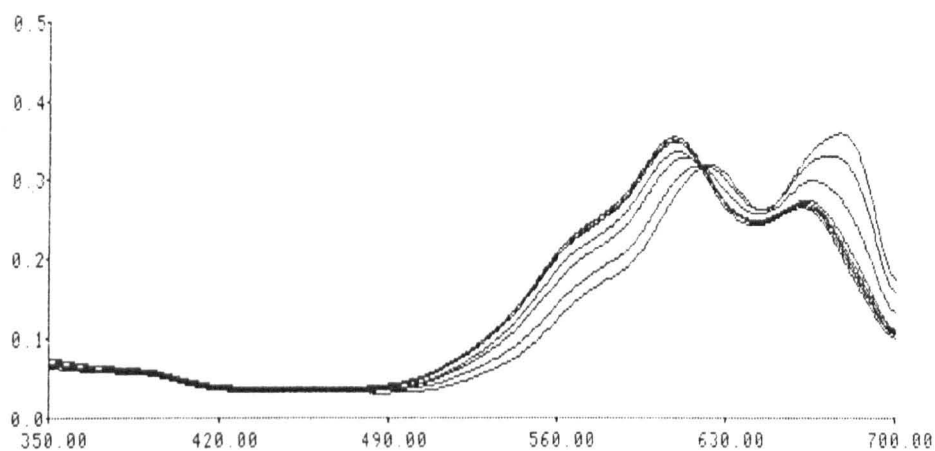


Fig 3. 2 Spectral shift in visible absorbance following the addition increasing concentrations of calf thymus DNA to 5,8-dihydroxyhydroxyethylaminoanthraquinone ZP275. Determined in high ionic strength buffer.

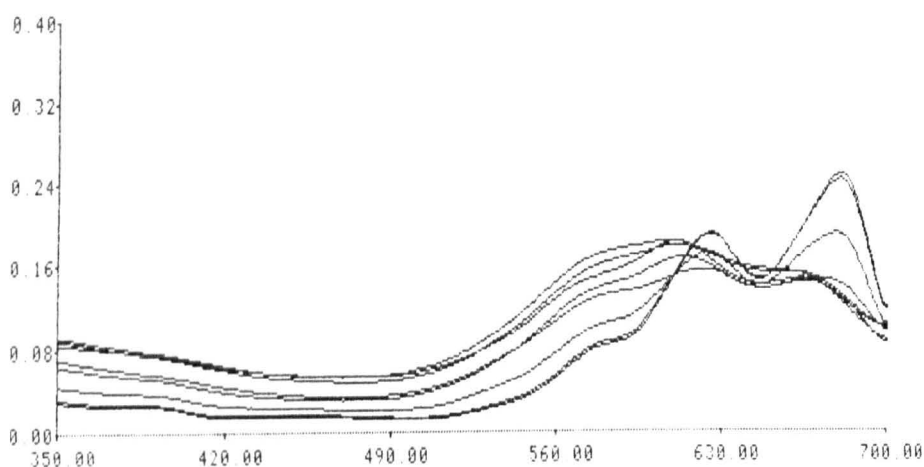


Fig 3. 3 Spectral shift in visible absorbance following the addition of increasing concentrations of calf thymus DNA to 5,8-dihydroxyhydroxyethylaminoanthraquinone ZP274. Determined in low ionic strength buffer.

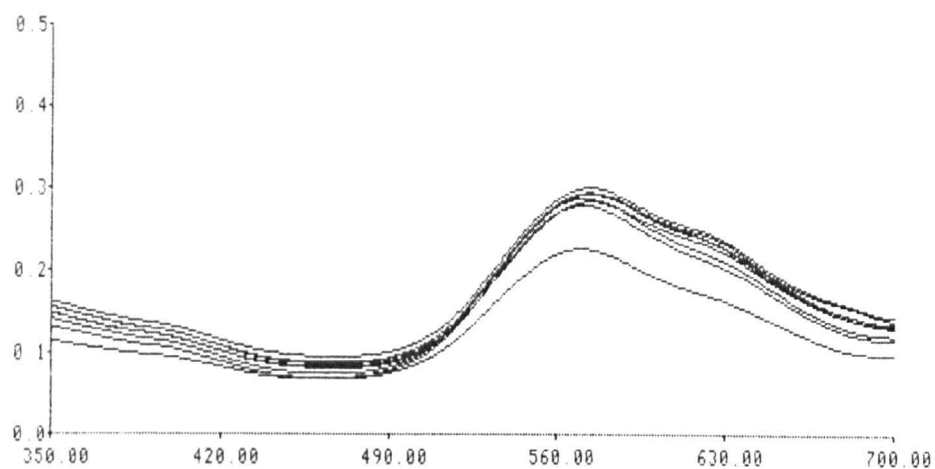


Fig 3. 4 Effect on visible absorbance following the addition of increasing concentration of calf thymus DNA to chloroethylamino-anthraquinone ZP285. Determined in high ionic strength buffer.

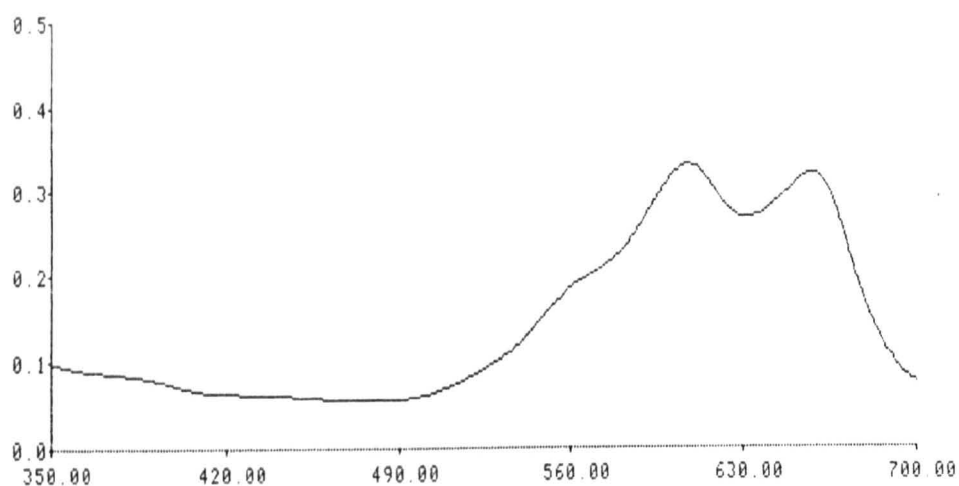


Fig 3. 5 The visible absorbance spectrum of ZP285 in dH₂O

3. 4. 2 SPECTROPHOTOMETRIC TITRATION STUDIES

In order to proceed with the spectrophotometric titration assay it is essential that each drug produces an isosbestic point (see section 3. 4. 1) and that both free and bound drug obey Beer-Lamberts Law over the drug concentrations used.

Following the addition of an increasing concentration of DNA to a fixed concentration of drug, intercalation can be identified by a decrease in absorbance at (λ_{\max}) of the free drug and a visible absorbance shift to longer wavelengths as a result of perturbation of the π and π^* orbital energies on binding. The extent and nature of DNA binding can be quantitated using the Scatchard equation which can be applied to intercalating species. By taking absorbance readings at the λ_{\max} of the free drug and plotting r/c vs r , where r = (concentration of ligand bound/concentration of DNA) and c = concentration of free ligand, the extent of DNA/drug association can be determined (Plumbridge *et al.*, 1978; Bell *et al.*, 1989). The negative slope corresponds to the affinity constant (K_1), whilst the fraction of drug molecule bound per DNA base pair (n) can be determined at the x intercept (Fig. 3. 6)

All titrations were performed in triplicate and in high ionic strength buffer to maintain the conditions of the drug isosbestic point determination where surface binding is excluded. At low concentrations of DNA, intercalative binding was shown to be negligible. Conversely, at high DNA/low drug concentration ratio intercalation predominates, and the graph tends towards a steep negative slope. The K_1 values for the 5,8-dihydroxyanthraquinone analogues were in the order of $10^6 M^{-1}$, whilst the K_1 values for the 5,8-dihydroanthraquinones were $10^6 - 10^7 M^{-1}$ (Table 3. 2).

The number of drug molecules bound per molecule of DNA (n) did not vary significantly amongst compounds of the same substituted chromophore. The 5,8-dihydroanthraquinones intercalated at every 8.3 base pairs of DNA ($n = 0.12$) whilst the 5,8-dihydroxyanthraquinones intercalated at approximately every 6.6 base pairs ($n = 0.15$).

The DNA affinity of the chloroethylaminoanthraquinones was not determined as the compounds did not show isosbestic behaviour (see section 3. 4. 1 and 3. 5).

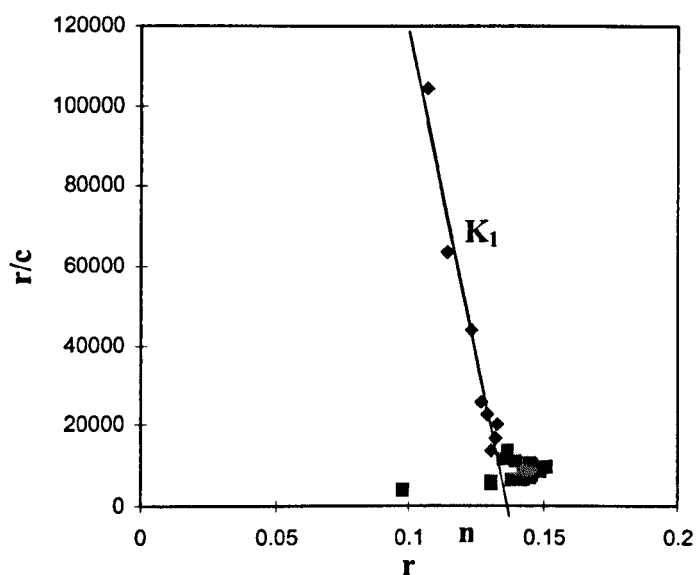


Fig. 3.6 Scatchard determination of the binding affinity of ZP265 with calf thymus DNA. The data shown represents the average of triplicate experiments. K_1 = drug/DNA association constant; n = fraction of drug molecule intercalated per base pair site

Table 3. 2 The affinity of the hydroxyethylaminoanthraquinones for calf thymus DNA.

Compound	$K_1(M^{-1})$	n
ZP232*	3×10^6	0.12 (8.3)
ZP240*	7×10^6	0.12 (8.3)
ZP242*	1×10^7	0.12 (8.3)
ZP255	3×10^6	0.15 (6.6)
ZP257	2×10^6	0.175(5.7)
ZP265	3×10^6	0.14 (7.1)
ZP274	2×10^6	0.15 (6.6)
ZP275	2×10^6	0.15 (6.6)

Data determined at the λ_{max} of drug. K_1 represents the DNA intercalation association constant and is expressed as the mean of three replicates; n is the fraction of drug molecule bound per DNA base pair and is expressed in parenthesis as the frequency of drug-base pair intercalation. * denotes 5, 8-dihydroanthraquinone.

3. 4. 3 DNA THERMAL DENATURATION TEMPERATURE STUDIES

The DNA melting temperature (T_m) was assayed according to the method of Tong *et al.*,(1978). T_m is defined as the temperature at which half of the concentration of DNA has denatured to single strands and can be derived by plotting absorbance vs temperature. By subtracting the T_m for DNA alone from the T_m for DNA intercalated with drug, the degree of helix stabilisation towards denaturation can be determined as ΔT_m .

All analysis was performed in triplicate in low ionic strength buffer. Calf thymus DNA with no drug gave a T_m value of 70.5 ± 0.5 °C.

The efficiency of helix stabilisation by the hydroxyethylaminoanthraquinones followed the general trend (least efficient) monoanthraquinones < 5,8-dihydroanthraquinones < 5,8-dihydroxyanthraquinones (Table 3. 3). The weakest drug proved to be monosubstituted ZP1 which showed poor binding with DNA, giving a ΔT_m of

0.4 °C. In contrast, the 1,4-disubstituted-5,8-dihydroxyanthraquinone ZP265, was the most effective drug, increasing DNA denaturation by 19.7 °C. Fig 3. 7 compares the effect of increased chromophore substitution on helix stability by showing the T_m plots for three drugs ZP1, ZP242 and ZP265 substituted with the (*N*-(2-hydroxyethyl)-*N*-methylamino)ethylenediamine sidechain (Fig. 3. 8). The effect of increasing the number of hydroxyethyl groups from one to two per side chain resulted in reduced DNA binding. This coincides with data derived from the isosbestic behaviour of ZP274 and the assumption that surface binding predominates with this compound. The evidence from the thermal denaturation studies of the hydroxyethylaminoanthraquinones therefore indicates that the chromophore 5,8 substituted hydroxyl groups and the structure of the side chain both affect the extent of drug-DNA binding.

The chloroethylaminoanthraquinones showed a general trend of weak helix interaction (Table 3. 4) suggesting that with the exception of ZP281 (ΔT_m 10. 1) (Fig 3. 11) they appear to have poor affinity for DNA. A possible explanation for this is discussed in (section 3. 5.)

Two compounds ZP254 and the chloroethylaminoanthraquinone ZP280 precipitated on addition to buffer and so determination of T_m was not possible.

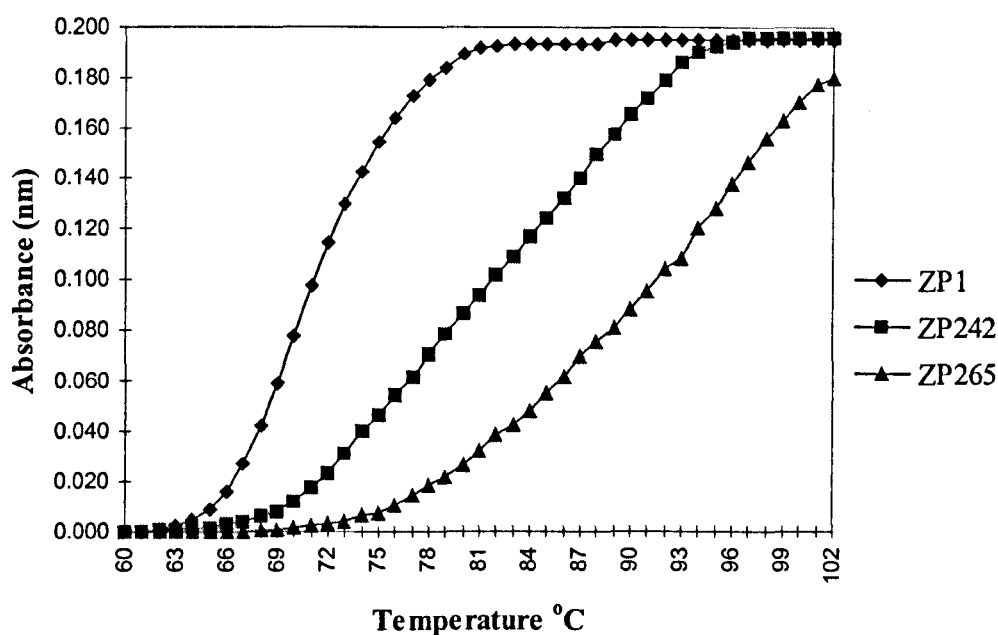


Fig. 3. 7 Demonstrates the effect of increasing temperature on the UV absorbance of calf thymus DNA (at λ_{260}) in the presence of ZP1, ZP242 and ZP265. The data shown for each compound is the average of triplicate experiments.

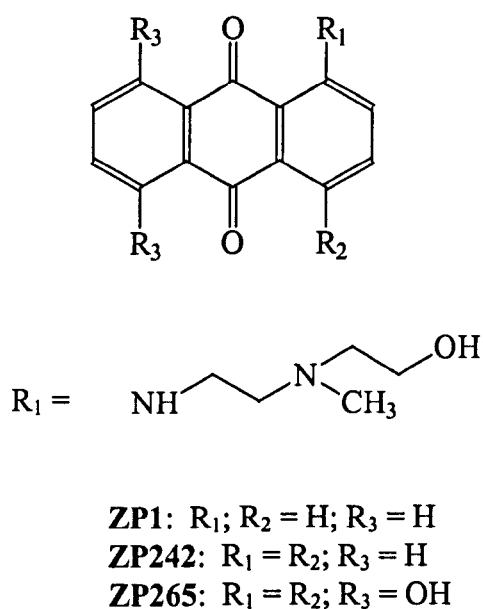


Fig 3. 8 Shows the chromophore side chain substitution pattern of ZP1, ZP242 and ZP265

Table 3. 3 The effect of hydroxyethylaminoanthraquinones on the denaturation temperature of calf thymus DNA.

Compound	T_m (°C)	ΔT_m (°C)
ZP1	70.9 (\pm 0.27)	0.4
ZP240	74.5 (\pm 0.21)	4.0
ZP242	81.7 (\pm 0.15)	11.3
ZP255	83.3 (\pm 0.35)	12.8
ZP257	80.1 (\pm 0.1)	10.5
ZP265	90.2 (\pm 0.22)	19.7
ZP274	81.5 (\pm 0.21)	11.0
ZP275	84.0 (\pm 0.32)	13.5

T_m determined at the λ_{260} of calf thymus DNA and expressed as the mean triplicate value (\pm s.d)

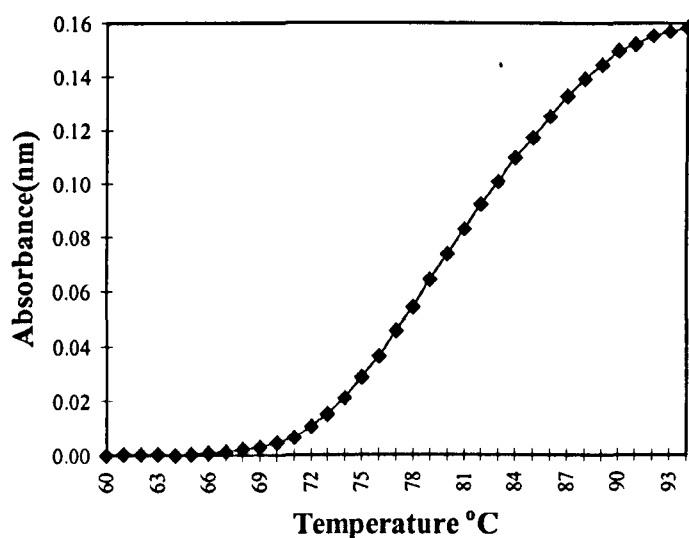


Fig 3. 9 The effect of increasing temperature on the UV absorbance of calf thymus DNA (λ_{260}) in the presence of chloroethylaminoanthraquinone ZP281. The data shown is the average value of triplicate experiments.

Table 3. 4 The effect of chloroethylaminoanthraquinones on the denaturation temperature of calf thymus DNA.

Compound	$T_m(^{\circ}\text{C})$	$\Delta T_m(^{\circ}\text{C})$
ZP293	72.2 (± 0.1)	1.7
ZP289	76.2 (± 0.53)	5.7
ZP286	77.7 (± 0.4)	7.2
ZP282	73.8 (± 0.41)	2.3
ZP285	75.9 (± 0.37)	5.4
ZP280	pption	n/a
ZP281	80.6 (± 0.41)	10.1

T_m determined at the λ_{260} DNA and expressed as the mean triplicate value (\pm s.d).
n/a = not applicable.

3. 5. DISCUSSION

3. 5. 1. ISOSBESTIC BEHAVIOUR OF THE NOVEL ANTHRAQUINONES

A property of substituted anthraquinones is to bind DNA by the process of intercalation which is manifest by a characteristic drug visible absorbance shift to longer wavelengths (bathochromic shift) and a decrease in the molar extinction at the λ_{max} of the free drug (hypochromic shift) (Waring *et al.*, 1981). When the drug becomes intercalated into DNA, it is stabilised within the hydrophobic interior of the helix by charge transfer forces, van der Waals and hydrophobic interactions. The effect of these are to alter the electron density of the chromophore and its absorbance properties. Anthraquinones containing basic amine side chains can also bind to DNA through ionic interaction. This is a weaker mode of binding compared to intercalation and occurs at physiological pH between the positively charged amino group on the sidechains and the anionic phosphate groups of the DNA backbone (reviewed by Baguely 1991, Pohle *et al.*, 1990).

Studies of the novel hydroxyethylaminoanthraquinones showed that 1,4-disubstituted 5,8-dihydro and the 5,8-dihydroxy but not the 1-substituted compounds gave isosbestic points in high ionic strength buffer (0.5M NaCl). The effect of high salt concentration is to neutralise the anionic charge of the DNA phosphate backbone with positively charged sodium ions and this discourages the drug from binding to the surface of the DNA but at the same time allows intercalative binding to continue (Lown *et al.*, 1985; Bell *et al.*, 1989). Evidence of electrostatic binding was apparent in low ionic strength buffer as the majority of the drugs lost isosbestic behaviour suggesting that significant surface binding had occurred. In view of this, the DNA binding affinity of the novel anthraquinones was determined in high ionic strength buffer. It is also worth noting that others have shown that the presence of an isosbestic point can be dependent on the buffer used (Kapuszcinski *et al.*, 1981; Lown *et al.*, 1985).

ZP274, comprising of two hydroxyethyl groups per side chain would only give a partial isosbestic point in low ionic strength buffer at the four lowest drug concentrations. It has previously been stated that intercalation is encouraged at a low drug to DNA ratio and the subsequent loss of an isosbestic point may be attributed to the ability of the drug to surface bind DNA at high drug/DNA ratios (Kapuszcinski *et al.*, 1985). The paucity of DNA binding sites may also encourage drug aggregation. Surface binding encourages 'self association' between drug molecules which stack on top of one another (dimerisation) through chromophore hydrophobic interactions and reduce the number of accessible intercalation sites. The outcome of this is to promote additional secondary binding and a species with absorption properties which differ from the intercalated drug (Kapuszcinski *et al.*, 1985). In the case of ZP274 it is possible that the partial isosbestic point occurred as a result DNA surface binding alone as at high ionic strength a complete loss of isosbestic behaviour was observed. From this evidence it can be postulated that intercalation as a single binding mode does not take place for this drug.

The mono substituted anthraquinones eg ZP1, ZP150 and ZP233 showed a lack of a consistent single interaction with DNA. Because they possess only one side chain this structural configuration allows for a number of intercalative binding modes to take place.

Islam *et al.*, (1985) demonstrated that 1-substituted anthraquinones can intercalate either parallel or perpendicular to the DNA axis with the sidechain in the major or minor groove of DNA and may therefore produce more than one DNA binding species in solution.

At high ionic strength the chloroethylaminoanthraquinones failed to show isosbestic behaviour. Instead at each DNA concentration they gave identical spectra which in contrast to the hydroxyethylaminoanthraquinones showed a large change in the visible region coinciding with a shift to shorter wavelengths (Fig. 3. 4). By comparison, at the same concentration of free drug in distilled water (no buffer or salt) the characteristic anthraquinone spectra was observed (Fig. 3. 5). A similar shift in spectrum profile was obtained in the theoretical calculation of mitoxantrone dimers (Kapuscinski *et al.*, 1985). Considering this, it may be postulated that high ionic strength promotes drug chromophore-chromophore hydrophobic interactions. These may lead to drug dimerisation and reduced DNA interaction, perhaps as a consequence of the high lipophilic nature of the chloroethylaminoanthraquinones. As a further suggestion, the lack of crossover points for spectra at high and low drug/DNA ratios does not necessarily indicate that intercalation did not take place because under certain conditions such as high ionic strength, free and bound drug may differ significantly to never give an isosbestic point.

3. 5. 2 ANALYSIS OF THE DNA BINDING CONSTANT (K_1) FOR THE HYDROXYETHYLAMINOANTHRAQUINONES

The determination of DNA affinity constants for the hydroxyethylaminoanthraquinones followed the method of Lown *et al.*, (1985); Bell *et al.*, (1989). This assay involves reverse titration where the DNA is added to and initially becomes saturated with drug. As DNA concentration increases, intercalation rather than surface binding predominates. Kapuscinski *et al.*, (1985) stated that this method can promote DNA condensation and precipitation which compromises accuracy, however, this effect was not observed in this study or that of Lown *et al.*, (1985).

The DNA association constant values (K_1) for the 1,4-disubstituted-5,8-dihydroxyanthraquinones were in the range 10^6M^{-1} at high ionic strength. This data is in

agreement with Lown *et al.*, (1985) who found that mitoxantrone and its congeners bound duplex DNA in the range $(0.16-2.35) \times 10^6 \text{M}^{-1}$, whilst the 5,8-dihydroanthraquinone derivatives such as ametantrone gave 10 fold lower values. The present study provided contrary results when the two classes of drug were compared as the 5,8-dihydroanthraquinones produced the higher (K_1) values (Table 3. 2) which is consistent with the data derived by the titration method of Kapuczinski *et al.*, (1985). Because DNA affinity is an equilibrium measure of the extent of drug attraction for DNA it may be affected by the choice of buffer or ionic concentration (Chaires *et al.*, 1982). This inconsistency has led others to suggest that the dissociation rate of a drug from DNA is a better indication of DNA affinity (Denny *et al.*, 1990).

When a comparison was made between the 1,4-disubstituted-5, 8-dihydro- and the 5,8-dihydroxyanthraquinones and the frequency of drug base pair intercalation (n) the latter were found to intercalate into DNA on average every 6.6 base pairs, an increase of approximately two base pairs over the 5, 8-dihydroanthraquinones. Previous studies by Lown *et al.*, (1985); Kapuczinski *et al.*, (1985) confirm this trend but demonstrated that mitoxantrone intercalates into DNA at approximately every 2.9 base pairs consistent with the neighbour exclusion model. This model suggests that the binding of one drug molecule to one site influences the binding of subsequent molecules by preventing binding at nearby sites either through physical blockage or by steric alterations of the DNA helix. Although the novel dihydroxyanthraquinones would be expected to have a similar intercalative binding frequency to mitoxantrone, it has been suggested that high ionic strength can reduce the number of binding sites by lowering the affinity of the drug for DNA (Chaires *et al.*, 1982).

From the DNA-drug affinity data it can be shown that the chromophore hydroxyl groups influence the equilibrium towards DNA-drug formation. This is particularly evident when comparing compounds with identical sidechains such as ZP242 and ZP65, and can be explained in a number of ways. The perpendicular mode of intercalation which is favoured by 1,4 disubstituted anthraquinones (see section 1. 3. 2. 2) orientates the chromophore hydroxyl groups such that they are able to penetrate the helix through to the

minor groove. The effect of this is to cause the disengagement of the chromophore from DNA to be energetically unfavourable and so considerably slower (Denny *et al.*, 1989). Prolonged chromophore retention times have also been attributed to the formation of intramolecular hydrogen bonds between the chromophore 5,8-hydroxyl groups and the quinone carbonyl groups. These bonding interactions increase the lipophilic character of the drug and its affinity for the hydrophobic interior of DNA (Patterson, 1993). Furthermore, the hydroxyl groups are suggested to introduce planarity to the chromophore, which is in contrast to ametantrone where the unsubstituted ring is slightly buckled. As a result the planarity of mitoxantrone has been suggested to enhance base pair interaction at the intercalation site (Mazerski *et al.*, 1998).

The consistency of data within each series of drugs i.e 5,8-dihydro or 5,8-hydroxyanthraquinone, shows that sidechain interactions play a minor role (if any) during the intercalation process at high ionic strength. The mode of action of the 5,8- dihydro and 5,8-dihydroxy-anthraquinones is therefore consistent with intercalative binding.

3. 5. 3. EFFECT OF THE HYDROXYETHYLAMINOANTHRAQUINONES ON STABILISATION OF THE DNA HELIX TO THERMAL DENATURATION

The DNA molecule is made up of two complementary strands consisting of a sugar phosphate backbone and purine/pyrimidine base pairs which are held together by hydrogen bonding. On heating, the DNA inter-strand hydrogen bonds break causing denaturation of the double helix which leads the formation of single-stranded DNA. Drug molecules that reversibly intercalate into DNA stabilize the helix such that more energy (a higher temperature) is required to cause denaturation. This effect can be measured by monitoring the change in absorbance with temperature at 260nm, the absorbance maximum for duplex DNA.

As all the drugs were assayed in low ionic concentration, it is therefore assumed that electrostatic binding in addition to intercalation contributes to the results obtained. The hydroxyethylaminoanthraquinones displayed a number of characteristics; drugs related by sidechain such as ZP1, ZP242 and ZP265 gave approximately 10°C rise in temperature

with each substitution onto the chromophore (Fig. 3. 7). The increasing order of drug-DNA stability followed the trend, mono substituted anthraquinone < 5,8-dihydroanthraquinone < 5,8-dihydroxyanthraquinone (both 1, 4-disubstituted). This is in agreement with the DNA dissociation kinetics of substituted anthraquinones (Gandecha *et al.*, 1985; Denny *et al.*, 1990). Drugs with two hydroxyethyl groups per sidechain such as ZP274, ZP240 and ZP255 demonstrated considerably reduced ΔT_m values. It is likely that the hydroxyethyl side chains of these compounds predispose them to increased hydrophilicity and preferential electrostatic binding to DNA. Since these interactions are external to the DNA duplex, low ΔT_m values would be expected.

1,4 disubstituted anthraquinones comprising of the (*N*-(2-hydroxyethyl)-*N*-methylamino)ethylenediamine sidechain gave the highest overall ΔT_m values (Fig. 3. 8 and Table 3. 3). ZP265 stabilised the helix by 19.7°C. In comparison, under similar conditions mitoxantrone stabilises DNA by 15.9 °C (Johnson *et al.*, 1979). At physiological pH the secondary amine side chains of mitoxantrone become protonated and interact with the negatively charged DNA-phosphate groups. Additional hydrogen bonding may occur between the drug side chain hydroxyl groups and the base pairs in the vicinity of the intercalation site (Bailly *et al.*, 1996; Mazerski *et al.*, 1998). In view of this, it is likely that ZP242 and ZP265 display a similar mode of base pair side chain interaction to mitoxantrone. Furthermore, as the tertiary methyl amine side chains increase drug lipophilicity they may essentially enhance DNA binding by making the drug more attractive to the DNA helix.

3. 5. 4. EFFECT OF THE CHLOROETHYLAMINOANTHRAQUINONES ON STABILISATION OF THE DNA HELIX TO THERMAL DENATURATION

At first sight, the data from chloroethylaminoanthraquinone-DNA interaction suggests that these compounds are weak, reversible DNA binding agents. This contrasts with the DNA alkylation studies of Chapter 4 which demonstrated that they inhibit DNA strand separation at nanomolar concentrations which is up to 100 fold less than in this assay. In view of this, it is likely that the formation of drug-DNA inter-strand crosslinks

takes place but too few crosslinks are created to stabilise the helix effectively. The consequence of drug crosslinking DNA is to prevent thermal denaturation (Hartley *et al.*, 1991), reducing the formation of single strand DNA rather than altering T_m and it can be directly linked to the number of crosslinks formed. Any hydrolysed drug would be expected to bind DNA non-covalently and determine the outcome of helix stability at the absorbance wavelength stated. Studies with nitrogen mustards such as melphalan have shown that the dihydroxyethylamine is the product of reaction under aqueous conditions (Bolton *et al.*, 1993). When fully hydrolysed the chloroethylaminoanthraquinones would be expected to form hydroxyethylaminoanthraquinones which have generally engendered good helix stability (Table 3. 3). These products of hydrolysis would probably be present in low concentration and so would have little effect on the helix stability. Additionally, mono- and perhaps intra-strand alkylations of the helix may take place (Osborne *et al.*, 1995). This form of covalent binding is unable to inhibit DNA strand separation and further to this may orientate the drug in such a way as to prevent it from intercalating into DNA.

Of the chloroethylaminoanthraquinones, ZP281 gave the highest ΔT_m (10.1 °C). The unsymmetrical nature of this compound restricts bis-alkylation of DNA to one side chain as its second arm is identical to the side chains substituted onto AQ4 (Fig. 2.12), a drug that has demonstrated high affinity for DNA (ΔT_m 17.5°C) (Patterson, 1993). Because ZP281 creates fewer inter-strand crosslinks (demonstrated in Chapter 4) it is likely to have a greater effect on DNA thermal melting temperature through the formation of a higher concentration of single-strand alkylations that are enhanced by the non-covalent interaction of its second side chain.

In summary, the hydroxyethylaminoanthraquinones showed good affinity for DNA consistent with both intercalative binding and ionic interactions. The chloroethylaminoanthraquinones with the exception of ZP281, demonstrated poor binding implying that intercalation may not be the primary mechanism by which these compounds interact with DNA. Results in Chapter 4 will suggest that rather than being poor DNA binders, the chloroethylaminoanthraquinones irreversibly alkylate DNA.

Chapter 4 DNA ALKYLATION STUDIES

4.1 INTRODUCTION

Rational anticancer drug development faces a dilemma that most drugs have multiple sites of action and it is not known which of these contribute to therapy or to toxicity. It is therefore beneficial to attempt to select compounds with a restricted range of action but without reduced anti-tumour activity. Bifunctional nitrogen mustards are extremely cytotoxic through their ability to form covalent intra and/or inter-strand crosslinks with DNA. However, their high reactivity and lack of target specificity often leads to reaction with other cellular nucleophiles and to a reduced concentration of drug reaching its target site (see section 1.4).

Nitrogen mustards linked to an acridine chromophore have previously demonstrated improved selectivity for DNA and an altered drug base pair specificity compared to non-targeted mustards (Mattes *et al.*, 1986). The novel chloroethylaminoanthraquinones are a series of drugs which have the capacity to both intercalate and alkylate DNA. To establish the extent of their covalent binding nature, the DNA base pair alkylation preference of these compounds and their ability to inter-strand crosslink DNA was determined.

4.2. CHEMICALS AND REAGENTS

Plasmid DNA pBR322 (0.25u/μl), T4 polynucleotide kinase (5u/μl), BAM H1 (10u/μl) and Sal I (10u/μl) restriction enzymes were purchased from Northumbria Biologicals Ltd. $\gamma^{32}\text{P}$ -ATP (500Ci/mmol), primer (10pm/μl), dNTP mix (2.5mM), MgCl_2 (25mM), gelatin (0.2%), Bacterial Alkaline Phosphatase (BAP) (150u/μl), Bacterial Alkaline Phosphatase reaction buffer (BAP buffer 5×), 10× buffer, React 3 buffer (10×), DNA stop solution, Forward reaction buffer (5×), were obtained from Amersham Biotech Ltd, St. Albans, Herts. Taq DNA polymerase (×50) (0.1u/μl) was purchased from Promega,

London BIO-Spin chromatography column and BIORAD 583 gel drier were obtained from BIO-RAD Laboratories, Kent, UK. Ammonium persulphate (APS), *N, N, N', N'*-tetramethylethylenediamine (TEMED), sequagel 6, complete mix, mineral oil and mechloroethamine (*N*-methyl-*N,N*-bis(2-chloroethyl)amine) were purchased from Sigma Chemicals, London, UK. The control mechloroethamine was made up to the appropriate concentration in 0.1M hydrochloric acid and stored at -20°C prior to use. The agents ZP281, ZP282, ZP285 and ZP289 were synthesised as described in section 2. 4, made up to the appropriate stock solution concentration in DMSO and stored at -20°C prior to use.

u = unit activity.

Strand separation buffer, TEoA buffer, TEA buffer, TBE buffer and formamide dye were all prepared according to appendix 2.

4. 3. METHODS

See appendix 1 for preparation of DNA

4. 3. 1 DETERMINATION OF DNA SEQUENCE SPECIFIC ALKYLATION USING THE POLYMERASE CHAIN REACTION (PCR)

The following constituents were added to an eppendorf containing the labelled primer (for preparation see appendix 1) and mixed: 0.2% gelatin (55µl), 25mM MgCl₂ (220µl), 10×buffer (110µl), 2.5nM dNTP mix (110µl) and dH₂O (55µl). The mixture (50µl) was placed into a PCR tube containing the drug-treated DNA (see appendix 1) (40µl) and the enzyme DNA Taq polymerase (×50) (10µl) which was diluted into dH₂O (1 in 50) prior to use. Each tube was covered with mineral oil and the PCR set to cycle 36 times at 94°C for 1 min, 58°C for 2 min, 72°C for 1 min and an additional 1 min per cycle, a total of 3 h. The ten samples were then transferred into sterile eppendorfs and the DNA precipitated and dried as described in appendix 1. Each dried sample was re-suspended in formamide dye (4µl), heated to 95°C for 2 min and cooled in an ice-bath to denature the DNA. The samples

were loaded onto the gel (see appendix 2) and electrophoresis was performed in TBE buffer over 3 h at 3000V, 55°C using vertical glass electrophoresis plates. The resulting gel was then transferred onto Whatman 3MM filter paper, covered and dried under pressure on a BIO-RAD 583 gel drier for approximately two hours. Once dry, the gel was exposed onto a Kodak X-ray film at -70°C for 24 h before developing.

4. 3. 2. DETERMINATION OF THE FORMATION OF DNA INTER STRAND CROSSLINKS

To each sample was added: ^{32}P -radiolabelled DNA (100ng/10 μl), drug: ZP281, ZP282, ZP285 or ZP289 (1 μl) in DMSO to give final assay drug concentrations of 0.1nM, 1nM, 10nM and 100nM, TEoA buffer (5 μl) and the total volume made up to 50 μl with dH₂O. Following incubation at 37°C for 1h the reaction was terminated by the addition of DNA stop solution (10 μl) to each sample. The DNA was precipitated by the addition of 95% ethanol (180 μl) and centrifuged at 1300rpm for 15 min. This precipitation process was repeated and the resulting pellet dried by lyophilisation. Each dried sample including the control sample of drug untreated DNA were resuspended in strand separation buffer (10 μl), heated at 95°C for 2 min in order to denature the DNA, then immediately chilled in an ice-water bath to prevent the DNA strands rejoining. A sample of undenatured DNA, the second control, was not heated, and instead was dissolved in formamide dye (10 μl). An 0.8% agarose gel approximately 1 cm thick was prepared in TEA buffer (also used as the running buffer) and the samples subsequently loaded. Electrophoresis was performed overnight using a horizontal gel kit at 40 V. The resultant gel was dried for 2 h at 95°C onto one layer of Whatman 3MM paper (covered in film wrap) in a BIO-RAD 583 gel drier connected to a vacuum. Autoradiography was performed using Kodak Medical X-ray film for 24 h at -70 °C.

4. 4. RESULTS

4. 4. 1 DNA SEQUENCE SPECIFIC ALKYLATION STUDIES

A synthetic 20 base oligonucleotide primer complimentary to bases 621-640 of the 273 base pair BamH I/Sal I fragment of pBR3222 (bases 375-650) was 5' end labelled and annealed to the DNA. Following extension with *Taq* DNA polymerase a full length fragment of 263 base pairs was produced. The alkylating concentration of each compound was determined by identifying the lowest level of modification per DNA fragment (usually one base pair) in order to enable qualitative comparisons to be made (Ponti *et al.*, 1991).

Four chloroethylaminoanthraquinones ZP281, ZP282, ZP285 and ZP289 were examined for their ability to block the progress of DNA *Taq* polymerase. Fig 4. 1 shows the result of drug alkylation at four assay concentrations 5nM, 10nM, 50nM and 100nM against full length DNA and a control, mechlorethamine (2.5 μ M). The unmodified DNA showed complete elongation with only a few sites of early termination. In contrast, the four alkylating anthraquinones showed inhibition of DNA elongation consistent with DNA alkylation at all concentrations tested.

All sites of alkylation occurred at guanine bases, consistent with previous studies of nitrogen mustards (Ponti, *et al.*, 1991). When alkylation patterns were compared between the targeted and non-targeted mustard mechlorethamine, a difference in guanine selectivity was observed (Table 4. 1 and Fig. 4. 1). The chloroethylaminoanthraquinones demonstrated a preference for guanines adjacent to a 5' pyrimidine (5'PyG), particularly isolated 5'PyGPy sites, which are sites of weak alkylation by nitrogen mustards (Kohn *et al.*, 1987). At the following sequences 5'-CGGGGGA-3' (bases 534-539); 5'PyGG (bases 506, 579 and 582) the chloroethylaminoanthraquinones preferentially alkylated at the 5'guanine base(s) whilst mechlorethamine selected the 3'side. At triplet guanine sequences 5'CGGGC (bases 471-473 and 486-488), the middle guanine was the most intense for all compounds and is consistent with the central guanine of a run of three being the most electronegative base (Pullman *et al.*, 1981).

Two distinguishing alkylation patterns separated the four compounds into two groups on the grounds of side chain length. These occurred at (bases 511-513 and 579-580). In the first instance, at the sequence 5' GGGT, ZP282 and ZP289 (both containing propylene side chains) alkylated preferentially at the 3' guanine, whilst no reaction was observed for ZP281 and ZP285. At the second sequence, 5'GG, both guanines were alkylated by ZP282 and ZP289, whilst ZP281 and ZP285 (containing an ethylene side chain) selectively alkylated the 5'guanine. Both examples are sites of relatively weak alkylation.

The concentration (5nM) of chloroethylaminoanthraquinone drug required to alkylate DNA was found to be 500 fold less than that for mechlorethamine (2.5 μ M) suggesting that the chloroethylaminoanthraquinones have high specificity for DNA. Furthermore the selection of 5'PyG sites implies that they have the potential to form a high degree of inter-strand crosslinks.

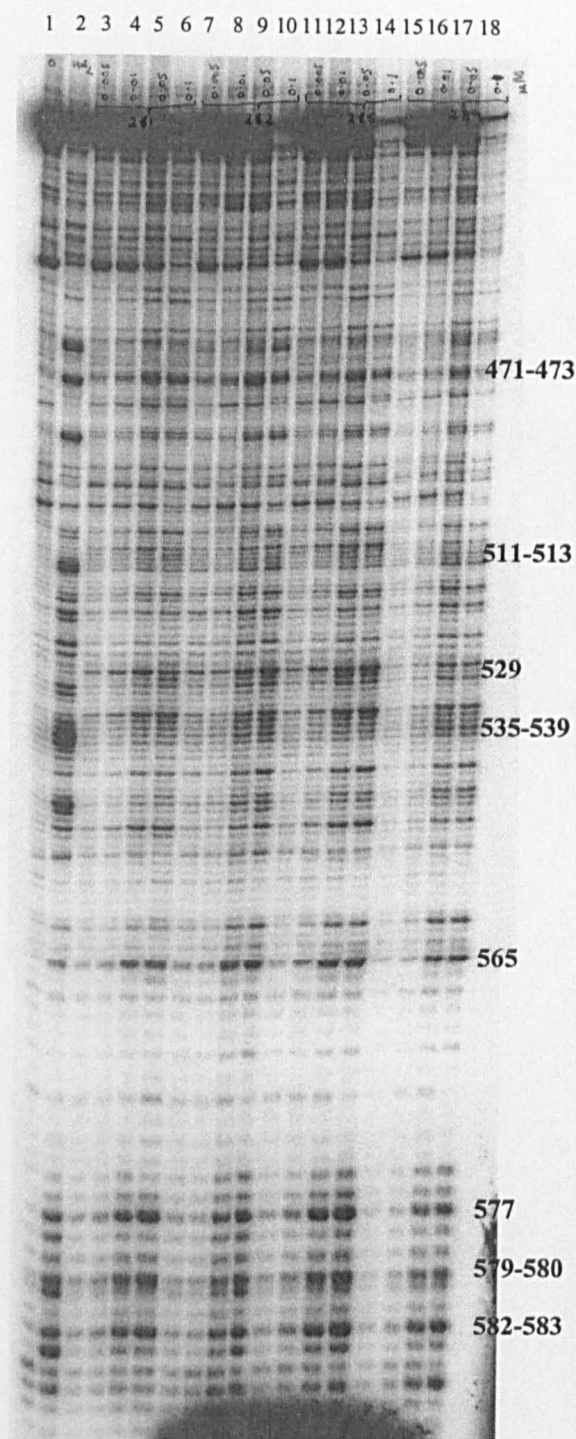


Fig 4.1 Gel electrophoresis of the sites of most frequent alkylation by the chloroethylantraquinones. Lane 1, unmodified DNA; Lane 2, Mechlorethamine (2.5 μ M); Lane 3-6, ZP281; Lane 7-10, ZP282; Lane 11-14, ZP285; Lane 15-18, ZP289. Increasing concentration of drug (left-right): 0.005, 0.01, 0.05 and 0.1 μ M

Table 4. 1 Sites of most intense alkylation by the chloroethylaminoanthraquinones and mechlorethamine at pBR322 DNA fragment (base pairs 375-650).

Alkylation site (bp. no)	Mechlor- ethamine	ZP281	ZP282	ZP285	ZP289
471-473	5' CGGGC	5' CGGGC	5' CGGGC	5' CGGGC	5' CGGGC
511-513	5' G GGT	nd	5' G GGT	nd	5' G GGT
529	nd	5' C GT	5' C GT	5' C GT	5' C GT
535-539	5' G GGGG	5' G GGGG	5' G GGGG	5' G GGGG	5' G GGGG
546-548	5' T GGGC	nd	5' T GGGC	5' T GGGC	5' T GGGC
550	5' C GC	5' C GC	5' C GC	5' C GC	5' C GC
565	nd	5' T GC	5' T GC	5' T GC	5' T GC
577	5' T GC	5' T GC	5' T GC	5' T GC	5' T GC
579-580	5' C GGC	5' C GGC	5' C GGC	5' C GGC	5' C GGC
582-583	5' C GGC	5' C GGC	5' C GGC	5' C GGC	5' C GGC

The highlighted 'G' denotes the sites of most reactivity by each compound. 'nd' denotes alkylation not detected. Bp.no (base pair number)

4. 4. 2 DNA INTER-STRAND CROSS LINKING STUDIES

The agarose gel assay is a reproducible method for the *in vitro* measurement of DNA inter-strand crosslinks. The principle of the reaction is that double stranded/or drug crosslinked DNA runs at a slower rate through agarose gel compared to denatured DNA which effectively runs as two mobile single strands. The extent of crosslinking can be calculated by quantification of the double and single-stranded DNA produced in each sample lane (Hartley *et al.*, 1991).

Linearised plasmid pBR322 DNA was reacted with increasing concentrations of drug 0.1nM, 1nM, 10nM, 100nM. By comparing drug treated DNA samples with the untreated controls which were single stranded (denatured) DNA, and double stranded DNA, the inhibition of DNA strand separation could be determined at each concentration for the chloroethylaminoanthraquinones ZP281, ZP282, ZP285 and ZP289. A dose dependent

increase in formation of inter-strand cross-links was observed with 100% cross-linking for each compound at the highest concentration of 100nM. ZP282 and ZP285 proved to be the most effective inter-strand cross-linking agents preventing over 50% strand separation at the lowest concentration 0.1nM. Whilst, ZP281 was the least effective creating mostly single stranded DNA at 0.1nM. The order of increasing reactivity was found to be ZP281 < ZP289 < ZP285 = ZP282 (most reactive). The data from this assay indicates that at the concentrations of drug used all of the compounds assayed have the ability to form inter-strand cross-links and may potentially be active as DNA cross-linking agents *in vivo*.

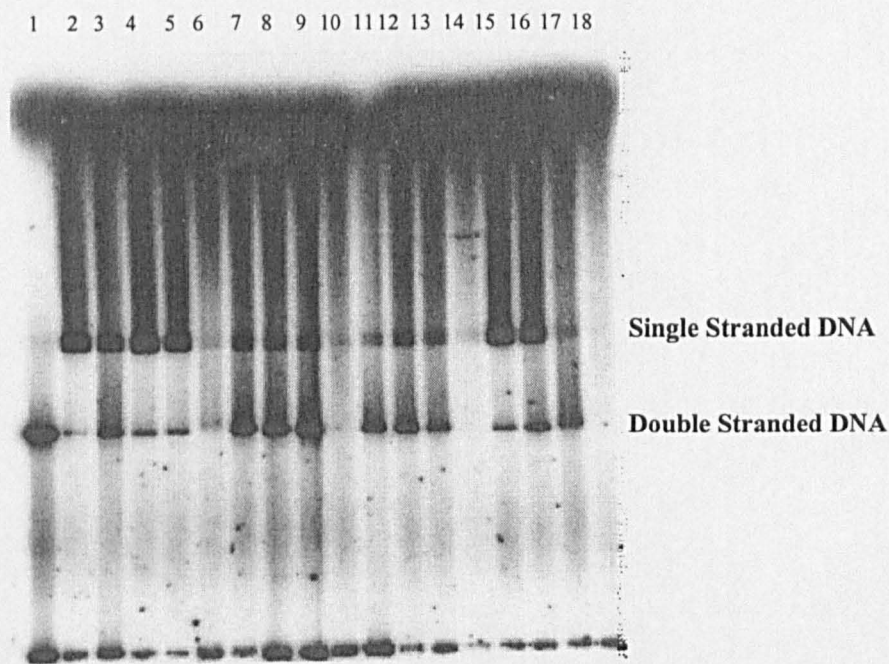
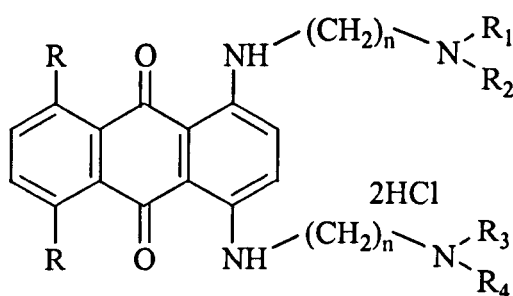


Fig 4. 2 Autoradiograph of the effects of chloroethylaminoanthraquinones on DNA strand separation inhibition. Lane 1, double stranded DNA, no drug; Lane 2, single stranded DNA; Lane 3-6, ZP281; Lane 7-10, ZP282; Lane 11-14, ZP285; Lane 15-18, ZP289. Increasing concentration of drug (left to right): 0.1, 1.0, 10.0 and 100nM

4. 5. DISCUSSION

4. 5. 1 IDENTIFICATION OF CHLOROETHYLAMINOANTHRAQUINONE-DNA SEQUENCE SPECIFIC ALKYLATION SITES

Nitrogen mustards such as mechlorethamine alkylate DNA through the formation of an aziridinium ion, a positively charged intermediate. By calculating the molecular electrostatic potential (MEP) of each base, Pulman *et al.*, (1981) identified that the most electronegative site on DNA is the nitrogen at the seven position of guanine (G N⁷) in the major groove. Furthermore, within contiguous guanines the central guanine was found to be the most electronegative and therefore the likely target of these agents. By use of gel electrophoresis techniques subsequent studies with various nitrogen mustards such as mechlorethamine and L-phenylalanine mustard (L-PAM) were able to confirm this evidence (Kohn *et al.*, 1987; Mattes *et al.*, 1986; Ponti *et al.*, 1991).



- ZP281** $n = 2$, $R = \text{OH}$, $R_1 = R_2 = -\text{CH}_2\text{CH}_2\text{Cl}$, $R_3 = R_4 = \text{CH}_3$
ZP282 $n = 3$, $R = \text{OH}$, $R_1 = R_2 = R_3 = R_4 = -\text{CH}_2\text{CH}_2\text{Cl}$
ZP285 $n = 2$, $R = \text{OH}$, $R_1 = R_3 = -\text{CH}_2\text{CH}_2\text{Cl}$, $R_2 = R_4 = \text{CH}_3$
ZP289 $n = 3$, $R = \text{H}$, $R_1 = R_2 = R_3 = R_4 = -\text{CH}_2\text{CH}_2\text{Cl}$

Fig. 4. 3. The structure of chloroethylaminoanthraquinones ZP281, ZP282, ZP285 and ZP289

Reaction of the chloroethylaminoanthraquinones ZP281, ZP282, ZP285 and ZP289 (Fig. 4. 3) with DNA showed that alkylation took place at most guanine bases but was most prominent at those adjacent to a 5'pyrimidine (cytosine or thymine). This was in contrast to mechlorethamine which generally preferred to alkylate the 3'guanine within a run of

guanines. This difference in base pair selectivity may be attributed to the influence of the DNA affinic anthraquinone chromophore. Altered sequence selectivity for DNA directed alkylating agents has been observed before. The reactivity of quinacrine mustard (Fig. 1.12) was found to depend on the two bases 3' to the reactive guanine and this is where intercalation was suggested to take place (Kohn *et al.*, 1987). This proposal was later amended when Kohn *et al.*, (1994) realised that such a model would impose major structural distortion of the DNA. Instead it was postulated that aliphatic mustards linked to a chromophore by a short spacer chain, typically 2-3 carbons, would most likely react with an immediately adjacent guanine on either side of the intercalation site depending on the flanking bases as these affect the reaction site conformation. By comparison the chloroethylaminoanthraquinones also contain short aliphatic spacer chains. Their preference for 5'PyG sites implies that intercalation may occur directly in between these two bases, with alkylation taking place at the 3' guanine.

To support the importance of the anthraquinone chromophore in DNA directed covalent binding, the DNA intercalation preference of mitoxantrone has been identified with 5'(A/T)CG triplet base pairs (Bailly *et al.*, 1996; Panousiss *et al.*, 1994). Two bases in this sequence correspond to the 5'PyG sites found for the mustard anthraquinones. Studies of acridine mustards have also suggested that the choice of chromophore may direct the site of alkylation. For example, 9-aminoacridine shows preference for intercalation at 5'PyPu base pair sites (Neidle *et al.*, 1984) and this correlates with the preferred 5'GCPu sites of alkylation by acridine mustard (Prakash *et al.*, 1990). Furthermore, the proposed mechanism of initial non-covalent binding followed by alkylation of nearby base pairs is consistent with that first postulated by Creech *et al.*, (1972).

Little variation existed between the alkylating patterns of the four novel drugs except at two sites. In both cases the same two compounds could be placed together in terms of sequence specificity, ZP282 and ZP289 containing three carbons and ZP281 and ZP285 containing two carbons. Previous studies have demonstrated the importance of the length of the side chain (see Table 4.2). For example, when aniline mustards linked to an acridine chromophore contained side chain linkers greater than three methylene groups in

length the alkylation specificity of these compounds changed from guanine to adenine (see Table 4. 2; Ferguson *et al.*, 1992). A series of short chain aliphatic mustards linked to acridine were found to be more DNA base pair specific than the longer side chain analogues which alkylated additionally at 5'GT sites (Kohn *et al.*, 1994). Short sidechains are thought to confer a degree of rigidity in the intercalation mode and can therefore restrict the number of accessible nucleophilic sites available for alkylation.

Table 4. 2 Summary of the preferred DNA alkylation sites determined for chromophore linked nitrogen mustards

Compound	Preferred base pair	
	alkylation site	Reference
Quinacrine mustard		
(s)	5'GG	Kohn <i>et al.</i> , 1986 Ponti <i>et al.</i> , 1991
(l)	5'GT	Kohn <i>et al.</i> , 1994
9- aminoacridine		
aniline mustard		
(s)	5'GT	Prakash <i>et al.</i> , 1990
(l)	5'AC	Ferguson <i>et al.</i> , 1992
Chloroethylamino- anthraquinones	5'PyG	

(s) denotes compounds with short linker chains. (l) denotes compounds with long linker chains >3.

Although the chromophore is thought to directly influence the site of covalent binding, it is likely that intercalation is not mandatory for alkylation to take place. Such reactions with DNA may perhaps be found at sites that are consistent with intense mechlorethamine binding.

Some of the most intense reactivity by the mustard anthraquinones occurred at lone guanines flanked either side by a pyrimidine base. Previous studies have demonstrated that

isolated 5'GC sequences are usually weakly reactive because the amino group of the adjacent cytosine exerts a positive electrostatic field in the vicinity of GN⁷ rendering it less electronegative (Kohn *et al.*, 1987). To date only the non-intercalating drug uracil mustard has shown selectivity for this site (Mattes *et al.*, 1986). In respect of this, it may be possible that the anthraquinone chromophore reduces the electrostatic influence of the adjacent cytosines and reinstates the electronegativity of the guanine making it more reactive. However, an alternative suggestion is that the high incidence of alkylation at these sites may result from intercalation taking place either side of the reactive guanine.

ZP289, was the most weakly reactive compound and this can be related to the lack of hydroxyl groups on the 5 and 8 position of its chromophore. These groups slow the rate of chromophore disengagement from DNA (Denny *et al.*, 1990) and consequently increase the probability of alkylation taking place at nearby base pairs.

Based on the lowest effective chloroethylaminoanthraquinone drug concentration used, the reaction concentration of quinacrine mustard (Ponti *et al.*, 1991) and mechlorethamine required to alkylate DNA was found to be 100 and 500 fold greater, respectively. In the first example, this may be a reflection of the weaker DNA affinity of the acridine chromophore compared to the anthraquinone chromophore (Feigon *et al.*, 1984; Denny *et al.*, 1989). Nevertheless, the important evidence to arise from this study is that the mustard anthraquinones have greatly enhanced selectivity for DNA over non-targeted mustards. Furthermore, reaction specificity at 5'PyG and isolated 5'PyGPy sites suggests that the chloroethylaminoanthraquinones are potentially selective for sites at which inter-strand crosslinks may be formed, that is 5'CG base pairs.

4. 5. 2. EFFECT OF CHLOROETHYLAMINOANTHRAQUINONES ON THE INHIBITION OF DNA STRAND SEPARATION

The anti-tumour activity of the nitrogen mustards is defined by their ability to form inter-strand and intra-strand crosslinks with DNA. Of these lesions, inter-strand crosslinks are considered to be the more relevant despite the fact that they make up only 4-7% of the total number of adducts formed by mechlorethamine (see section 1. 4)(Kohn *et al.*, 1966).

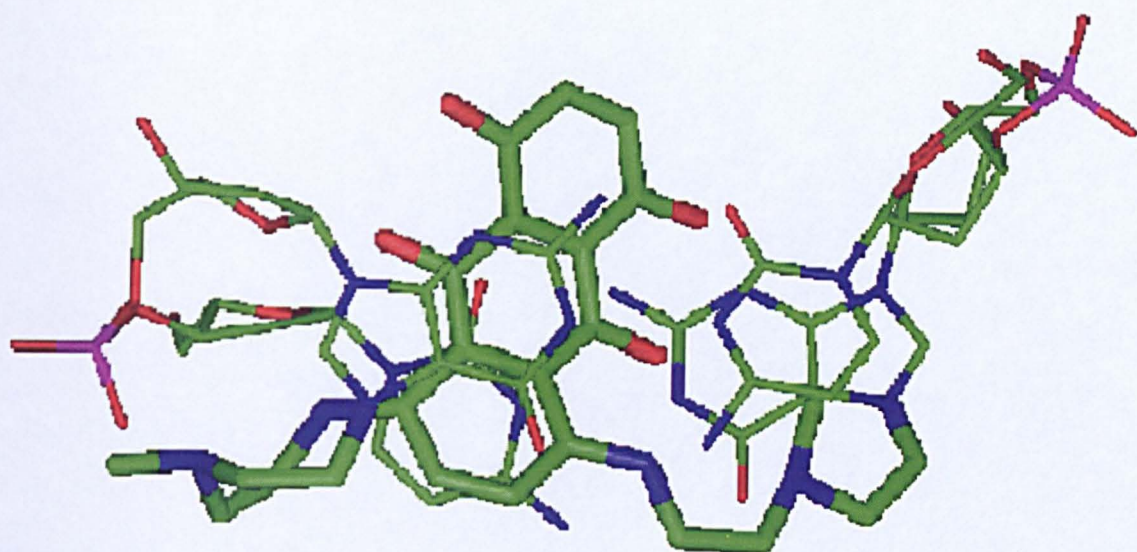


Fig. 4. 4 A representation of ZP285 intercalated into and forming an inter-strand crosslink between a 5'CG base pair

The mustard anthraquinones were able to inhibit DNA strand separation with varying degrees down to sub-nanomolar concentrations. This confirms the earlier observation (see section 4. 5. 1) that the anthraquinone chromophore plays an important role in directing the mustard function towards DNA and potential sites of inter-strand crosslink formation through 5'PyG base pair selectivity. Studies with aniline mustard attached to various acridine chromophores have also demonstrated greatly improved inhibition of strand separation over their respective untargeted parent mustard (Prakash *et al.*, 1990).

The order of increasing reactivity of the chloroethylaminoanthraquinones was found to be ZP281 < ZP289 < ZP285 = ZP282. This reflects the fact that ZP281 contains only one alkylating sidechain and so may preferentially react with one strand of DNA to form a greater number of intra-strand crosslinks. These do not prevent strand separation. In contrast, ZP282, ZP285 and ZP289 are capable of alkylating each DNA strand with separate side chains which predisposes them to a greater range of nucleophilic sites on DNA and

hence an increased chance of inter-strand crosslink formation. Despite this, ZP281 is an extremely effective inter-strand crosslinking agent suggesting that the bifunctional nature of its mustard side chain is sufficient in length to alkylate across two strands of DNA.

Early studies of DNA sequence specific alkylation identified 5'GC sequences as sites of inter-strand crosslink formation (Mattes *et al.*, 1986). More recently, it has been shown that 5'GNC (N=any base) complementary strands are the actual sites of crosslink formation for nitrogen mustards such as mechlorethamine and L-PAM (Osborne *et al.* 1993, Rink *et al.*, 1993). To achieve this crosslink the DNA duplex has to bend and distort. It can be argued that if intercalation takes place previous to DNA alkylation the DNA bases will become separated a further 3.4 angstroms (Lerman, 1961) to accommodate the anthraquinone chromophore, and this increase in distance may prevent alkylation across three base pairs. Further to this, it has been noted that compounds with a short linker chain are restricted to reaction at nearby nucleophilic sites (Kohn *et al.*, 1994) indicating that 5'GC or 5'CG inter-strand crosslinks are more likely to result. However, since the alkylating groups of bis-mustard 1,4-disubstituted anthraquinones are far more widely spaced than either mechlorethamine or quinacrine, this new class of compound is not necessarily restricted to this paradigm.

In summary, the sequence specific DNA studies have demonstrated that the chloroethylaminoanthraquinones alkylate DNA at guanine bases. They show selectivity for 5'PyG and isolated 5'PyGPy sites and have considerably improved DNA targeting over mechlorethamine. This is thought to be directly related to the presence of a DNA affinic anthraquinone chromophore within their structure. Furthermore, the sequence preference of these agents inferred that they would be good candidates for inter-strand crosslink formation and this was confirmed by the low concentration of drug that was required to inhibit DNA strand separation. The two studies have identified that the chloroethylaminoanthraquinones are excellent DNA inter-strand crosslinking agents whose mechanism of action may take place by initial DNA intercalation followed by the alkylation of adjacent base pairs.

Chapter 5 TOPOISOMERASE II INHIBITION STUDIES

5.1 INTRODUCTION

Topoisomerase II (topo II) is essential for cell proliferation and cell viability, hence it is an attractive target for anti-cancer agent development. Unlike mammalian cells, yeast cells possess a single topo II enzyme which presumably performs the functions of both human isoforms (Nitiss *et al.*, 1988). Recent studies have shown that the yeast topo II can successfully be replaced with either of the human topo II isoenzymes, α or β , which can then be expressed from full length cDNA in a drug permeable yeast strain such as *Saccharomyces cerevisiae* (*Sc.ce*) (Hammonds *et al.*, 1998). In view of this, each isoenzyme may be studied individually without the effects of the second isoform interfering with data. Moreover, the topo II selectivity and isoenzyme specificity of a drug can be assessed more accurately.

As topo II is the cellular target for a number of intercalating agents including the drug mitoxantrone (section 1. 3. 2), compounds of an analogous structure would also be expected to target this enzyme. Three novel hydroxyethylaminoanthraquinones (ZP257, ZP265 and ZP275) and four chloroethylaminoanthraquinones (ZP281, ZP282, ZP285 and ZP289) were assayed to determine their topo II inhibitory activity and isoform selectivity in relation to drug covalent and non-covalent binding.

5.2. CHEMICALS AND REAGENTS

Yeast Nitrogen Base (YNB) purchased from Gibco, UK. -Leucine (-LEU) drop out media was purchased from Bio 101, UK. Mineral Oil (nuclease free), biological grade DMSO and glucose were purchased from Sigma, Poole, UK. Agar was purchased from Biogene Ltd, UK. All materials, yeast strains and plasmids were kindly supplied by Dr J. Jenkins, MRC Toxicology Unit, Leicester, UK. All compounds were serially diluted into

DMSO and stored at -20°C prior to use. Following the addition of drug to media the final drug concentrations were 0.1 µM, 1µM, 5µM, 10µM, 50µM and 100µM.

5. 3. CHARACTERISATION OF TOPO II α AND β YEAST STRAIN

The yeast used in this assay was the JJ700 strain expressing a promoter from the following plasmids: pYTO300 (wild-type *Saccharomyces cerevisiae* (*Sc. ce*) topo II gene); pHT300 (human topo II α gene [cDNA]); pHT400 (human topo II β gene [cDNA]). Yeast cells were constructed and grown in synthetic media by Dr J. Jenkins, MRC Toxicology Unit, Leicester, UK.

5. 4. DETERMINATION OF THE EFFECT OF ANTHRA-QUINONES ON YEAST STRAINS CONTAINING HUMAN α , β and *SC.CEREVISAE* TOPOISOMERASE II

The three yeast strains (deletion strains (Δ topo2)) containing either plasmid pHT300, pHT400 or pYTO300 were grown at 30°C on agar plates containing medium lacking leucine and 2% glucose. A small sample was taken from the growing colonies of each strain and diluted into the synthetic media (see appendix 2) to produce seeding cultures containing approximately 10^5 to 10^6 cells/ml which gave an OD₆₃₀ reading (optical density at 630nm) of 0.05. To 96 microtitre plate wells was added the media (174µl), the drug (1µl) in DMSO at six different concentrations, or DMSO (1µl) (control), and finally the yeast seeding culture (25µl). Each well was overlayed with 2 drops (approximately 50µl) of mineral oil and initial OD₆₃₀ readings were taken before leaving the cultures to grow at 30°C. Following a twenty hour incubation period the well contents were carefully mixed with a multi-channel pipette, and the OD₆₃₀ recalculated for each well. All experiments to determine the 50% inhibitory concentration (IC₅₀) for each compound were performed in triplicate.

5. 5 RESULTS

5. 5. 1. EFFECT OF NOVEL ANTHRAQUINONES ON YEAST STRAIN GROWTH

The growth inhibition of topo II isoform containing yeast cells was determined by the assay of (Hammonds *et al.*, 1998). Values were obtained by calculating cell survival as a percentage of the control OD₆₃₀ and plotted against six increasing drug concentrations for each topoisomerase II (α , β and *Sc.ce*) containing yeast strain. Furthermore, as the anthraquinone chromophore absorbs in the visible region at 630nm it was also necessary to subtract the OD₆₃₀ of drug in media from the appropriate drug containing yeast cells to give the actual cell survival .

Three chloroethylaminoanthraquinones ZP281, ZP285 and ZP289 inhibited the growth of yeast containing either: human topo II α , human topo II β or wild type *Sc.ce* topo II. The increasing order of activity was (least active) ZP289 < ZP281 < ZP285 (Table 5.1). ZP285, demonstrated the highest inhibitory activity at approximately (IC₅₀ 4 μ M) for all three yeast strains, and similar to ZP281 in that both showed no isoenzyme specificity (Fig. 5. 1 and Fig. 5. 2). In comparison, ZP289 showed a slight preference for the α isoenzyme. ZP282 was anomalous to the trend of topo II inhibition established by the chloroethylaminoanthraquinones, and was found to be inactive in all three yeast strains at the highest concentration. The reason for this is unknown.

The hydroxyethylaminoanthraquinones ZP257, ZP265 and ZP275 had no effect on cell viability at low concentrations and precipitated from the media at 100 μ M which prevented a conclusive assessment of their topo II activity from being made.

All drugs were assayed in triplicate and the standard error at each concentration point was determined for the three active compounds (Fig. 5. 1-5. 3). By applying the Student's T-test (Carter *et al.*, 1981) at the 5% single tail significance level it was established that the data did not conclusively state that ZP281, ZP285 or ZP289 target human topo II α or β selectively. The standard error for the three compounds was 1.25, 1.32 and 0.94 respectively which is below the 5% confidence level of 2.16 at four degrees of freedom.

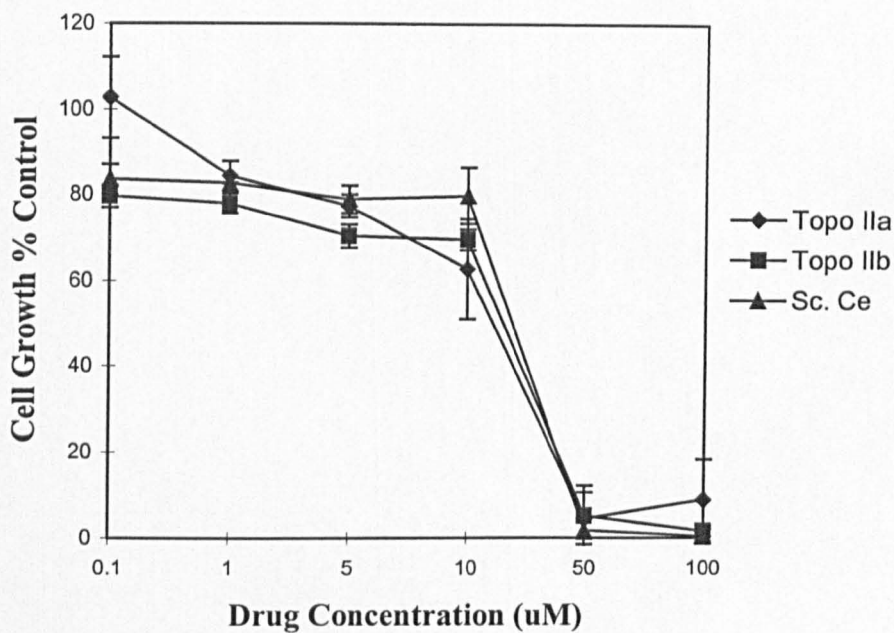


Fig 5.1 Effect of ZP281 on the cell survival of yeast transfected with human topo II α , β and yeast topo II. All samples were determined at OD₆₃₀ and given as mean triplicate values with standard deviation at each concentration point.

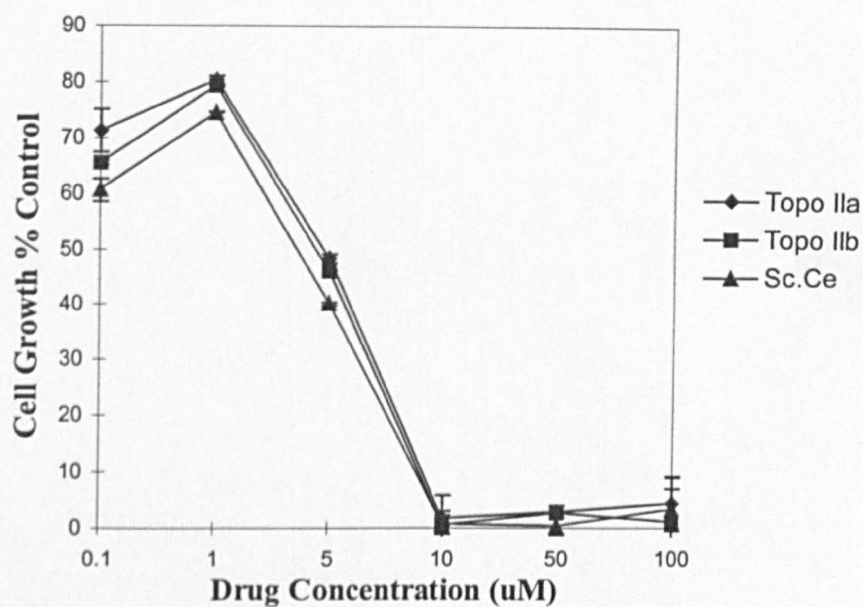


Fig 5. 2 Effect of ZP285 on the cell survival of yeast transfected with human topo II α , topo II β and yeast topo II. All samples measured at OD₆₃₀ and given as mean triplicate values with standard deviation at each concentration point.

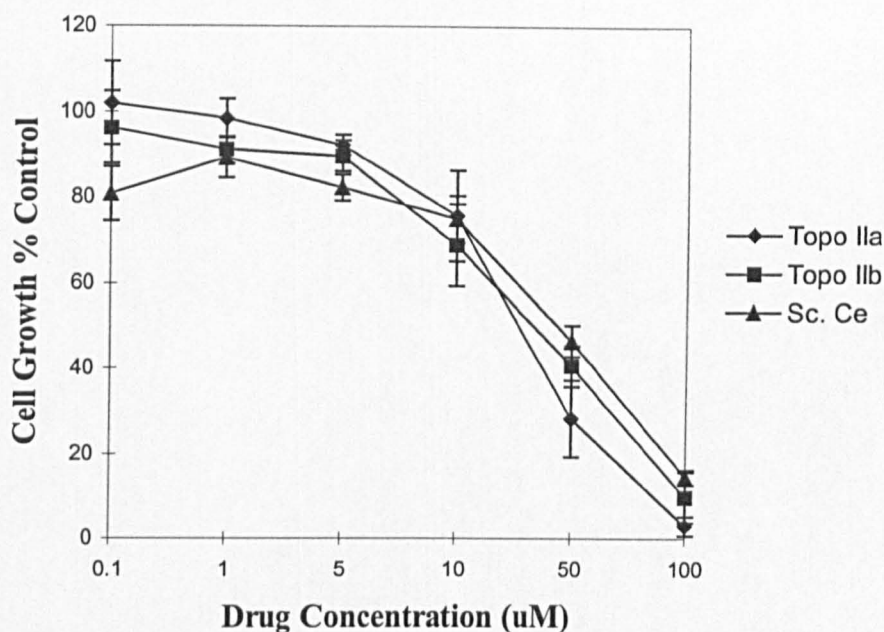


Fig 5. 3 Effect of ZP289 on the survival of yeast cells transfected with human topo II α , β or yeast topo II. All samples measured at OD₆₃₀ and given as mean triplicate values with standard deviation at each concentration point.

Table 5.1 The effect of the chlorethylaminoanthraquinones on the inhibition of yeast cell growth

	Topo II α	Topo II β	Topo II
DRUG	IC ₅₀ (μ M)	IC ₅₀ (μ M)	IC ₅₀ (μ M)
ZP281	24.7 \pm 2.5	21.9 \pm 1.9	25.1 \pm 2.1
ZP282	n/i	n/i	n/i
ZP285	4.4 \pm 0.1	4.3 \pm 0.02	3.8 \pm 0.05
ZP289	36.4 \pm 9.9	45.9 \pm 14.7	47.6 \pm 8.1

Topo II α and β represent plasmid DNA containing either human isoform α or β ; topo II represents plasmid DNA containing yeast topo II from *Saccharomyces Cereviciae*. IC₅₀(μ M) = concentration of drug required to inhibit 50% of cell growth (\pm standard deviation, n = 3); n/i=no inhibition

5. 6. DISCUSSION

Topo II is a DNA associated enzyme which introduces a double-strand break into a DNA segment allowing the passage of another duplex through the break before resealing the cleavage. Many Topo II inhibitors are thought to induce cytotoxicity by stabilizing the DNA-topo II cleavable complex and preventing strand religation, they are known as 'topo II poisons' (Fig. 1. 2B).

Investigation into the effects of seven novel anthraquinones on the inhibition of yeast transfected with either human topo II α , human topo II β or yeast topo II (*Sc. ce*) demonstrated that three chloroethylaminoanthraquinones ZP281, ZP285 and ZP289 (Fig. 4. 3) may potentially inhibit this enzyme. Their increasing order of reactivity was (least potent) ZP289 < ZP281 < ZP285. Suprisingly, ZP282 was not cytotoxic towards any of the yeast strains. This is contrary to studies in Chapter 4 which suggested that the chloroethylaminoanthraquinones including ZP282 selectively crosslinked DNA through a chromophore directed mechanism of alkylation. Furthermore, in mammalian cells ZP282 was found to be extremely potent demonstrating growth inhibition and a cross-resistance profile similar to the most cytotoxic chloroethylaminoanthraquinones (see Chapter 6). Evidence therefore suggested that ZP282 would also inhibit yeast cell growth. The reason for this lack of cytotoxicity in yeast, even at high drug concentration, is unknown.

Computer modelling has indicated that compounds that share the same relative space occupancy and electronic distribution, even if chemically unrelated, have the same topo II-DNA sequence specificity (Paoletti *et al.*, 1993). Furthermore, it is recognised that nucleotides immediately adjacent to the site of Topo II/DNA cleavage are specific to this class of inhibitor. For example, in the presence of mitoxantrone, topo II cleaves DNA with a pyrimidine base 5' (Paoletti *et al.*, 1993; Capranico *et al.*, 1993) and this site shows consistency with the preferred DNA alkylation sequence identified for the chloroethylaminoanthraquinones, 5'PyG (see Chapter 4). By analogy, the cellular mechanism of topo II inhibition by these agents is likely occur in a similar if not identical

manner to mitoxantrone, the difference being that the chloroethylaminoanthraquinones would bind covalently to their target.

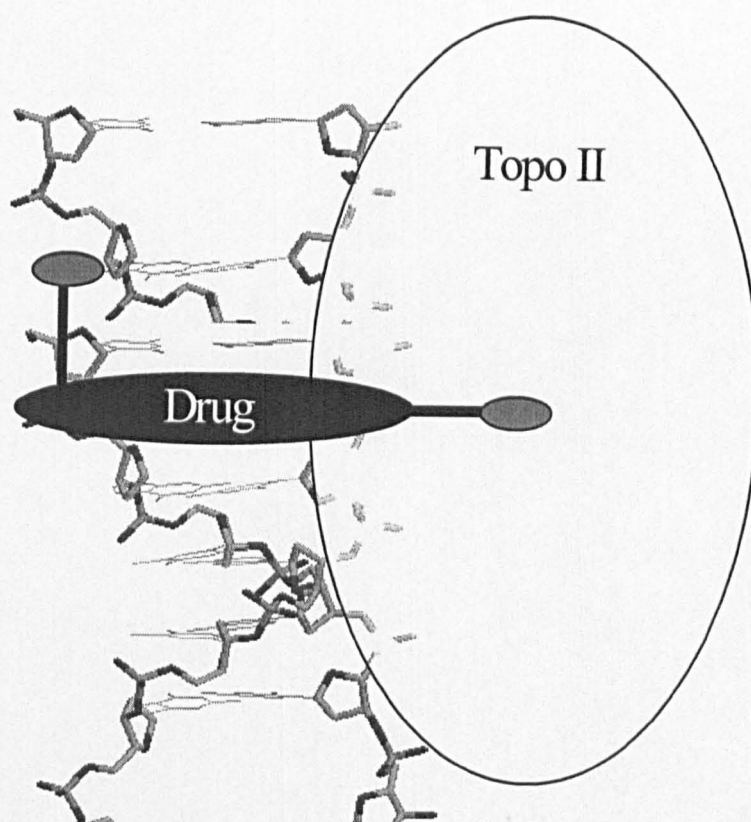


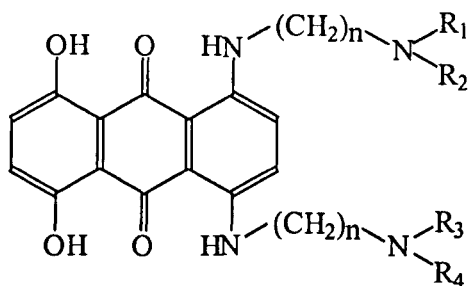
Fig. 5. 4 Shows the drug crosslinked with topo II and DNA to form a ternary complex

Most topo II inhibitors associate with the cleavable-complex by non-covalent binding and so the drug is often readily displaced from the active site, particularly if cellular resistance mechanism are present (see section 1. 5). Conversely, drugs that have the capacity to intercalate into DNA (Fig. 5. 4) and form covalent DNA-protein crosslinks should remain permanently trapped at the cleavage site. This would lead to an irreversible inhibition of strand religation and to increased DNA double strand break production which is vital to the onset of cell death. An investigation into the mechanism of topo II inhibition

has identified a model in which the drug forms a ternary complex with the DNA /enzyme and is placed at the interface between the DNA cleavage and enzyme active sites (Capranico *et al.*, 1990). To substantiate this, a photoactivatable mAMSA analogue has demonstrated covalent binding to DNA bases at the +1 and -1 positions of the cleavage site (Freundreich *et al.*, 1994). Moreover, camptothecin is proposed to interact at specific amino acid residues on topo I and with its GC specific base pair sequence at the topo I/ DNA active site (Reindbo *et al.*, 1998). Collectively these studies support the idea that covalent inhibitors such as the chloroethylaminoanthraquinones which may target the topo II/DNA active site have the potential to alkylate specific nucleophilic sites on the enzyme and/or DNA following initial non-covalent association with DNA.

Various groups have found that enzyme inhibition for a number of topo II targeted agents can be dependent on a particular isoform and can also influence the outcome of drug resistance (see section 1. 5. 1. 2). In the yeast assay of Hammonds *et al.*, (1998) mitoxantrone inhibited topo II α at half the concentration required to inhibit topo II β . A similar conclusion was derived by Meczes *et al.*, (1997) using temperature sensitive yeast strains. Of the drugs that showed inhibitory activity in this study only ZP289 demonstrated a slight preference for a particular enzyme isoform and this was topo II α . By close inspection of the data the large standard deviation at the drug (IC₅₀) make this observation inconclusive (Table 5. 1). In contrast, ZP285 and ZP281 did not distinguish between either isoenzyme which was evident by the similarity in their cell growth inhibition curves (Fig. 5.1 and Fig. 5. 2). As a consequence it is not possible to state conclusively that they inhibit topo II and may indeed have a different cellular target. However, previous reports of indiscriminate isoform selection have been observed for doxorubicin (Hammonds *et al.*, 1998) and m-AMSA (Meczes *et al.*, 1997) and can perhaps be reasoned by the high sequence homology of topo II α and β , even within the active site (Austin *et al.*, 1993). It is possible that such an effect would be more pronounced with the mustard anthraquinones because of the potential for drug covalent binding of nucleophilic sites within the active region of the enzyme.

The hydroxyethylaminoanthraquinones ZP257, ZP265 and ZP275 (Fig 5. 5) precipitated at high concentration (100 μ M) and at low concentrations had no effect on cell growth inhibition. DNA binding studies (see Chapter 3) demonstrate that these compounds have strong affinity for DNA in a manner which is consistent with intercalative binding. They are also extremely cytotoxic and have shown cross-resistance with doxorubicin in an ovarian



ZP257	n = 2, R ₁ = -CH ₂ CH ₂ OH; R ₂ = R ₃ = R ₄ = CH ₃
ZP265	n = 2, R ₁ = R ₃ = -CH ₂ CH ₂ OH, R ₂ = R ₄ = CH ₃
ZP275	n = 2, R ₁ = R ₂ = -CH ₂ CH ₂ OH, R ₃ = R ₄ = CH ₃

Fig 5. 5 Structure of ZP257, ZP265 and ZP275

carcinoma cell line expressing *MDR1* which confers resistance to natural products such as the anthraquinones (see Chapter 6). Furthermore, mitoxantrone an analogous drug (see Fig. 1. 5) has demonstrated both potency and enzyme isoform selectivity in an identical assay (Hammonds *et al.*, 1998). Hence, it was expected that the hydroxyethylaminoanthraquinones would also target topo II. One explanation for poor drug activity in yeast is low cell permeability through the yeast cell wall. Mutations are required to be placed in specific yeast genes so that sufficient drug is absorbed and this is reflected by the higher concentration of drug required to kill yeast cells compared to mammalian cells (Nitiss, 1994). It has been suggested that to enhance drug uptake of these agents it may be necessary to use a different permeability mutation to the *ISE2* (Inhibitor Sensitive 2) gene which is present in this yeast (personal comm. Dr J. R Jenkins). Alternatively, the

high cytotoxicity of the chloroethylaminoanthraquinones may result from a lack of DNA repair enzymes. Mutations in genes that abolish DNA repair such as *RAD52* are necessary to promote yeast cell kill following the creation of double strand breaks resulting from topo II inhibition (Nitiss, 1994). These mutations may also serve to enhance the cytotoxicity of the nitrogen mustards as such drugs have previously demonstrated increased sensitivity when cellular DNA repair mechanisms are absent (section 1. 5. 1. 4)

In summary, this study demonstrated that three compounds ZP281, ZP285 and ZP289 inhibited yeast cell growth at low concentrations, possibly as a consequence of topo II enzyme inhibition. When specificity was investigated, ZP281 and ZP285 did not exhibit a preference for either isoform which may be explained by the high sequence homology between the active sites of the isoenzymes. In contrast, yeast containing ZP282 and the non-mustard analogues resulted in no measurable loss of yeast viability. This may be due to poor drug penetration into the yeast or to efficient lesion repair. In conclusion, the data suggests that the DNA targeted nitrogen mustard anthraquinones have greatly enhanced cytotoxicity towards yeast when compared to their hydroxyethylaminoanthraquinone counterparts.

Chapter 6 CYTOTOXICITY STUDIES

6. 1 INTRODUCTION

The success of the quinone anti-cancer agents over the past 30 years has led to the synthesis of structurally related compounds in an attempt to enhance antitumour activity and potentially avoid the mechanisms of resistance that are associated with the prolonged use of these drugs in the clinic (section 1. 5.). A series of chloroethylamino- and hydroxyethylaminoanthraquinones have been synthesised on the principle that the alkylating anthraquinones may induce irreversible DNA damage by covalent stabilisation of the topo II-DNA cleavable complex. As the hydroxyethylaminoanthraquinones would principally act by non-covalent means, a difference in cytotoxicity and cellular resistance could be predicted.

This study was performed in two wild type ovarian cancer cell lines A2780 and CH1 and their cisplatin or adriamycin resistant mutants A2780CisR, CH1cisR, SKOV-3 and 2780AD. Resistance was also determined in chromosome transfer modified cisplatin resistant cell line B1.

6. 2 METHODS

6. 2. 1. DETERMINATION OF CYTOTOXICITY OF ANTHRAQUINONES IN WILD TYPE AND CISPLATIN RESISTANT HUMAN OVARIAN CANCER CELL LINES

The cytotoxicity (IC_{50}) values of substituted anthraquinones in five human ovarian carcinoma cell lines: A2780, A2780cisR, CH1, CH1cisR and SKOV-3 were determined by Dr Lloyd R Kelland, CRC Centre for Cancer Therapeutics, Sutton. The compounds were dissolved into DMSO at a concentration of 20mM immediately before adding to the cell lines to give final drug concentrations of 25, 5, 1, 0.25 and 0.05 μ M. Cell growth inhibition

was determined after 24h drug exposure followed by a 3 day growth period and assessed by staining with sulphorhodamine B (SRB) and measuring the remaining cell numbers. Seven compounds ZP257, ZP265, ZP275, ZP281, ZP282, ZP285 and ZP289 were selected for re-evaluation at lower concentrations at 4h drug exposure and 24 h drug exposure.

6. 2. 2. COMPARATIVE STUDY OF CYTOTOXICITY IN WILD TYPE, CISPLATIN RESISTANT AND ANTHACYCLINE RESISTANT HUMAN OVARIAN CANCER CELL LINES

Cytotoxicity (IC_{50}) was further investigated with ovarian carcinoma cell lines A2780 and 2780CP (also known as A2780CisR or CP70) both assayed in section 6. 2. 1, in addition to 2780AD and B1 by the CRC Department of Medical Oncology, Beatson Laboratories, University of Glasgow. Drug sensitivity was determined by the MTT assay with a 24 hour drug exposure period and 3 day growth period.

6. 3 RESULTS

6. 3. 1. EFFECT OF SUBSTITUTED ANTHRAQUINONES ON A2780, A2780cisR, CH1, CH1cisR and SKOV-3 CELL LINE VIABILITY

The cytotoxicity of a series of hydroxyethyl- and chloroethylaminoanthraquinones was investigated in wild type ovarian carcinoma cell lines A2780 and CH1, their cisplatin resistant cell lines A2780cisR and CH1cisR and also in SKOV-3, an ovarian cell line that shows intrinsic resistance to cisplatin (Table 6. 1 and Table 6. 2). A range of *in vitro* drug potency was observed across all cell lines assayed in some cases at nM concentrations. Identification of a relationship between compound structure and cytotoxicity revealed that in most cases the 1,4-disubstituted-5,8-dihydroxyanthraquinones were the most potent. The order of increasing cytotoxicity was found to be mono substituted anthraquinones < 5,8-dihydroanthraquinones < 5,8-dihydroxyanthraquinones. This trend was observed within the hydroxyethyl and the chloroethylaminoanthraquinones classes of drug.

Alkylating anthraquinones generally exhibited greater potency over their hydroxyethyl equivalent compounds. The relative difference in cytotoxicity was dependent on the drug and the cell line used. For example in A2780, ZP289 was approximately 110 fold more cytotoxic than ZP240. Monofunctional alkylating agents were also found to be less potent than their difunctional equivalents and this is exemplified by ZP288 which was approximately 5 fold less cytotoxic than ZP284.

Resistance factors ($RF = IC_{50} \text{ resistant cell line} / IC_{50} \text{ parent cell line}$) were found to be below 3 for all compounds assayed demonstrating low cross-resistance against cisplatin. ZP281 was the least cross resistant agent ($RF \text{ approx. } 1$) in all cell lines.

After 24hr drug exposure seven compounds ZP257, ZP265, ZP275, ZP281, ZP282, ZP285, ZP289 inhibited cell growth below the concentration of $0.05\mu\text{M}$. This assay was subsequently repeated in the same cell lines at lower drug concentrations and an additional comparative assay was performed to determine the effects of cell growth after short term (4h) drug exposure. In tables (6. 3 and 6. 4.) the data from these assays is compared with historical values derived for mitoxantrone, cisplatin, chlorambucil and doxorubicin under the same conditions. By prolonging drug exposure in non resistant cell lines (4h to 24h) a 2-5 fold increase in potency was observed. In the resistant cell line A2780cisR the cytotoxicity of ZP257 and ZP265 was increased by 200 fold after 24hrs. In contrast only a 6-8 fold increase in cytotoxicity was observed for the alkylating anthraquinones in the same cell line. This effect was specific to A2780cisR as in CH1cisR all compounds produced a similar increase in cytotoxicity. Only ZP245 was inactive at the highest drug concentration ($25\mu\text{M}$).

By comparison of historical data it can be shown that seven anthraquinone compounds were more potent cytotoxins than the antitumour drugs mitoxantrone, cisplatin and chlorambucil in all the cell lines assayed.

Table 6. 1 The effect of 24h drug exposure on the growth of ovarian cancer cell line A2780

Compound	A2780 IC ₅₀ (μ M)	A2780cisR IC ₅₀ (μ M)	RF
ZP1	3.4	4.2	1.2
ZP150	3.1	3.3	1.1
ZP232	3.3	3.5	1.1
ZP240	3.5	3.4	1.0
ZP242	0.72	0.58	0.8
ZP245	>25	>25	-
ZP249	3.3	3.2	1.0
ZP254	6.6	19.0	2.4
ZP255	0.68	0.74	1.1
ZP257	<0.05	<0.05	-
ZP265	<0.05	<0.05	-
ZP274	0.082	0.115	1.4
ZP275	<0.05	<0.05	-
ZP280(M)	0.12	0.17	1.4
ZP281(M)	<0.05	<0.05	-
ZP282(M)	<0.05	<0.05	-
ZP284(M)	0.41	0.64	1.6
ZP285(M)	<0.05	<0.05	-
ZP286(M)	0.135	0.355	2.6
ZP288(M)	2.7	2.7	1.0
ZP289(M)	<0.05	<0.05	-
ZP290(M)	0.051	0.096	1.9
ZP293(M)	4.6	8.0	1.7

A2780 is the wild type ovarian cell line; and A2780cisR is its cisplatin resistant cell line. RF=Resistance factor; IC₅₀ concentration of drug(μ M) required to inhibit 50% of cell growth. M=chloroethylaminoanthraquinone

Table 6. 2 The effect of 24h drug exposure on the growth of ovarian cancer cell lines CH1 and SKOV-3.

Compound	CH1 IC ₅₀ (μM)	CH1cisR IC ₅₀ (μM)	RF	SKOV-3 IC ₅₀ (μM)
ZP1	2.9	2.8	1.0	13
ZP150	2.6	2.6	1.0	11.5
ZP232	3.9	3.3	0.8	18
ZP240	2.2	2.4	1.1	6.7
ZP242	0.68	0.8	1.2	3.3
ZP245	>25	>25	-	>25
ZP249	2.4	2.3	1.0	9.2
ZP254	13	11.0	0.9	21.5
ZP255	0.58	0.78	1.3	2.45
ZP257	<0.05	<0.05	-	0.3
ZP265	<0.05	<0.05	-	11
ZP274	0.3	0.37	1.2	1.5
ZP275	<0.05	<0.05	-	0.26
ZP280(M)	0.054	0.11	2.0	1.45
ZP281(M)	<0.05	<0.05	-	0.155
ZP282(M)	<0.05	<0.05	-	0.185
ZP284(M)	0.185	0.55	2.9	1.9
ZP285(M)	<0.05	<0.05	-	0.1
ZP286(M)	0.11	0.145	1.3	0.77
ZP288(M)	1.44	1.3		10.8
ZP289(M)	<0.05	<0.05	-	0.19
ZP290(M)	0.078	0.092	1.2	0.86
ZP293(M)	2.5	3.1	1.2	13.5

CH1 is the wild type ovarian cell line; and CH1cisR is its cisplatin resistant cell line. SKOV-3 is intrinsically resistant ovarian cell line. RF=Resistance factor; IC₅₀ concentration of drug (μM) required to inhibit 50% of cell growth. M=chloroethylaminoanthraquinone

Table 6. 3 The cell growth inhibition concentration of the seven most potent anthraquinone compounds following 24h exposure to wild type A2780 and cisplatin resistant ovarian carcinoma cell line A2780CisR.

Compound	A2780(IC ₅₀)	A2780cisR(IC ₅₀)	RF
	(μ M)	(μ M)	
ZP257	0.005	0.0058	1.0
ZP265	0.0041	0.0039	1.0
ZP275	0.0125	0.013	1.0
ZP281(M)	0.016	0.019	1.2
ZP282(M)	0.0275	0.0375	1.4
ZP285(M)	0.009	0.024	2.7
ZP289(M)	0.031	0.052	1.7
Mitoxantrone	0.92	0.93	1.0
Cisplatin	0.3	3.3	11
Chlorambucil	9.0	52.5	5.8
Doxorubicin	0.01	0.014	1.4

IC₅₀ represents the concentration of drug required to inhibit cell growth by 50 %. RF = Resistance factor (ratio of inhibition in the resistant cell line/ inhibition in parent cell line). (M)=chloroethylaminoanthraquinone

Table 6. 4 The cell growth inhibition concentration of the seven most potent anthraquinone compounds following 24h exposure to wild type CH1 and cisplatin resistant CH1CisR and SKOV-3 ovarian carcinoma cell lines.

Compound	CH1 IC ₅₀ (μ M)	CH1cisR IC ₅₀ (μ M)	RF	SKOV-3 (μ M)
ZP257	0.0235	0.045	1.9	0.6
ZP265	0.0082	0.0215	2.6	0.24
ZP275	0.0295	0.046	1.6	0.85
ZP281(M)	0.018	0.019	1.0	0.49
ZP282(M)	0.025	0.035	1.4	0.76
ZP285(M)	0.0069	0.0135	1.9	0.29
ZP289(M)	0.03	0.11	3.7	0.82
Mitoxantrone	0.54	>1.0	-	>1.0
Cisplatin	0.1	0.65	6.5	4.4
Chlorambucil	3.3	7.9	2.4	35
Doxorubicin	0.0035	0.004	1.1	0.05

IC₅₀ represents the concentration of drug (μ M) required to achieve 50% inhibition of cell growth. RF=resistance factor; IC₅₀ resistant cell line/IC₅₀ parent cell line. M=chloroethylaminoanthraquinone

Table 6. 5 The effect of short term (4 hour) drug exposure on the growth of human ovarian carcinoma cell line A2780 and resistant cell line A2780cisR

Compound	A2780 IC ₅₀	A2780cisR IC ₅₀	RF
	(μ M)	(μ M)	
ZP257	0.089	1.45	16.3
ZP265	<0.05	0.78	-
ZP275	0.06	0.15	2.5
ZP281(M)	<0.05	0.28	-
ZP282(M)	0.195	0.22	1.1
ZP285(M)	0.052	0.17	3.3
ZP289(M)	0.165	0.4	2.4
Mitoxantrone	12.5	12.5	1.0

RF=Resistance Factor; M=chloroethylaminoanthraquinone

Table 6. 6 The effect of short term (4 hour) drug exposure on the growth of human ovarian carcinoma cell line CH1, cisplatin resistant cell lines CH1cisR and SKOV-3.

Compound	CH1 IC ₅₀	CH1cisR IC ₅₀	RF	SKOV-3 IC ₅₀
	(μ M)	(μ M)		(μ M)
ZP257	0.063	0.35	5.5	2.6
ZP265	<0.05	0.17	-	0.9
ZP275	0.074	0.58	7.8	2.1
ZP281(M)	0.058	0.41	7.1	0.66
ZP282(M)	0.175	0.85	4.8	2.8
ZP285(M)	<0.05	0.16	-	0.64
ZP289(M)	0.165	0.82	4.9	2.2
Mitoxantrone	5.1	12.0	2.3	>25

IC₅₀ is the concentration of drug required to inhibit cell growth by 50%. RF=resistance factor; IC₅₀ resistant cell line/ IC₅₀ parent cell line. M=chloroethylaminoanthraquinone

6. 3. 2. THE EFFECT OF SUBSTITUTED ANTHRAQUINONES ON A2780, 2780AD, A2780CisR(CP70) AND B1 CELL LINE VIABILITY

The cytotoxicity of seven compounds ZP257, ZP265, ZP275, ZP281, ZP282, ZP285 and ZP289 were investigated in the following human ovarian carcinoma cell lines supplied by a different laboratory: Wild type A2780 and cisplatin resistant cell line 2780CP (also known as A2780CisR/or CP70), both previously assayed in section 6. 2. 1. 1. Doxorubicin resistant cell line 2780AD and chromosome transfer modified cell line B1 (Table 6. 7 and Table 6. 8). Included are the cytotoxicities of cisplatin and doxorubicin assayed in the same cell lines.

The data highlighted mechanisms of resistance that distinguished the alkylating anthraquinones ZP281, ZP282, ZP285 and ZP289 from the three hydroxyethyl anthraquinones. All compounds, with the exception of ZP289, were found to be more potent than doxorubicin in the parent cell line A2780. ZP265 and ZP285 a hydroxyethylaminoanthraquinone and chloroethylaminoanthraquinone respectively, gave IC_{50} values of less than 1 nM. All compounds showed varying degrees of cross-resistance in the anthracycline resistant cell line 2780AD. ZP285 was the most cross resistant (RF 616) whilst ZP281 exhibited only a 4-fold resistance.

As expected, mechanisms that are responsible for cisplatin resistance such as those present in A2780CisR had little effect on the cytotoxicity of the hydroxyethylaminoanthraquinones. RF values for these compounds were similar to doxorubicin (RF 2.6) and in agreement with cell growth inhibition studies in the same cell lines demonstrated in section 6. 3. 1. In contrast, three chloroethylaminoanthraquinones ZP282, ZP285 and ZP289 all showed approximately 16-fold cross-resistance with cisplatin, whilst ZP281 again demonstrated weak cross- resistance (RF 2.9).

In B1, the re-introduction of the mismatch repair hMLH1 gene (reviewed in section 1. 5. 1. 4) resulted in only a 40-70% restoration of the original cytotoxicity of the chloroethylaminoanthraquinones suggesting that mismatch repair is potentially a mechanism which contributes to the cell sensitivity of these agents.

Table 6. 7 Effect of 24hr drug exposure on the growth of human ovarian carcinoma cell line A2780 and doxorubicin resistant cell line 2780AD.

Compound	A2780 IC ₅₀ (nM)	2780AD IC ₅₀ (nM)	RF
ZP257*	1.69 ± 0.14	40.4 ± 6.6	23.9
	1.57 ± 0.39	17.1 ± 1.0	10.9
ZP265	0.71 ± 0.32	21.2 ± 1.5	30
ZP275*	1.12 ± 0.14	144.5 ± 7.5	129
	1.42 ± 0.48		
ZP281(M)	4.01 ± 0.57	16.0 ± 1.72	4.0
ZP282(M)*	3.04 ± 0.57	245.8 ± 11.8	81
	4.47 ± 0.46	308.7 ± 2.3	69
ZP285(M)	0.93 ± 0.31	573.3 ± 132.6	616
ZP289(M)	7.56 ± 0.88	2637 ± 154	348
Cisplatin	340.0 ± 0.06	3.02 ± 0.22	9.0
Doxorubicin	4.98 ± 1.07	4860 ± 510	976

* compounds evaluated on two separate occasions to confirm the initial data. M=chloroethylaminoanthraquinone; IC₅₀ represents the concentration of drug (nM) required to inhibit 50% cell growth. ± = standard error of the mean triplicate estimations. RF=resistance factor.

Table 6. 8 Effect of 24hr drug exposure on the growth of human ovarian carcinoma cell line 2780CP and chromosome transfer modified cell line B1.

Compound	2780CP IC ₅₀		B1 IC ₅₀	
	(nM)	RF	(nM)	RF
ZP257*	1.22 ± 0.45	1.0	0.93 ± 0.17	1.0
	1.68 ± 0.22	1.0	2.20 ± 0.12	1.4
ZP265	1.55 ± 0.24	2.1	1.79 ± 0.24	2.5
ZP275*	2.55 ± 0.44	2.2	2.19 ± 0.65	2.0
	3.98 ± 0.73	2.8	5.02 ± 0.42	3.5
ZP281(M)*	11.8 ± 1.4	2.9	7.02 ± 0.64	1.7
	50.2 ± 3.9	16.5	19.6 ± 2.6	6.4
ZP282(M)	76.0 ± 12.8	25	42.9 ± 4.1	9.5
ZP285(M)	14.3 ± 1.0	15.3	5.81 ± 1.53	6.2
ZP289(M)	125.9 ± 20.8	16.6	66.0 ± 4.5	8.7
Cisplatin	2.62 ± 0.73	7.7	1.83 ± 0.23	5.3
Doxorubicin	13.3 ± 0.9	2.6	11.7 ± 1.0	2.3

* compounds evaluated twice to confirm initial data. M=chloroethylaminoanthraquinone
IC₅₀ represents the concentration of drug (nM) required to inhibit 50% cell growth.

± = standard error of the mean triplicate estimations; RF=resistance factor (ratio of IC₅₀ resistance cell line/ parent cell line A2780)

6.4. DISCUSSION

The human ovarian carcinoma cell line was selected for this study as anthracyclines such as doxorubicin and the anthraquinone mitoxantrone are often used in combination with cis-diamminedichloroplatinum (II) (cisplatin) in the clinical treatment of ovarian cancer. Although treatment is initially successful, it can often fail due to acquired resistance to these agents. Cisplatin causes cytotoxicity by platinating DNA. It is generally classed alongside the alkylating agents as it is able to form both DNA inter and intra-strand

crosslinks at the guanine N⁷ position (Eastman *et al.*, 1983). Furthermore, the development of cross-resistance amongst anti-cancer agents generally indicates similar mechanisms of toxicity.

All the drugs tested showed excellent activity in all cell lines (Table 6. 1 - 6. 8). The cytotoxicity of drug was found to increase in the order 1-monosubstituted anthraquinone < 1, 4-disubstituted-5,8-dihydroanthraquinone < 1, 4-disubstituted-5,8-dihydroxyanthraquinone for both the chloroethylamino- and the hydroxyethylaminoanthraquinone classes of compound. A similar relationship was established from the DNA affinity studies of the hydroxyethylaminoanthraquinones (Chapter 3), and can be related to both sidechain and hydroxyl group substitution onto the chromophore. Early cytotoxicity studies of 1, 4-disubstituted anthraquinones demonstrated that mitoxantrone which contains 5 and 8 substituted chromophore hydroxyl groups is up to 100 fold more potent than ametantrone which is unsubstituted (Nishio *et al.*, 1983).

Generally, the mustard anthraquinones were found to be the more potent compounds. This can be explained by their ability to bind covalently with their target (see Chapter 4) which is suggested to lead to the creation of permanent cytotoxic lesions. In contrast, the non-covalent binding nature of the hydroxyethylaminoanthraquinones predisposes them to cellular efflux over time, particularly if resistance mechanisms are present (Smith *et al.*, 1990 and 1992). Previous reports have indicated that nitrogen mustards that have an ability to bis-alkylate DNA tend to be more cytotoxic than their monoalkylating counterparts. Kohn *et al.*, (1994) observed that cytotoxicity increased 10 fold when comparing mono to bifunctional alkylating 9-aminoacridines in human carcinoma HT-29 cells. The reduced potency of the monofunctional alkylating agents, including the mono alkylating chloroethylaminoanthraquinones, is related to their inability to form DNA crosslinks as they are instead thought to form mono adducts which are considered to be genotoxic rather than cytotoxic (Povirk *et al.*, 1994).

Seven compounds ZP257, ZP265, ZP275, ZP281, ZP282, ZP285, ZP289 were re-assayed at nanomolar concentrations and demonstrated a comparable cytotoxicity to doxorubicin in all cell lines. Consistent with mitoxantrone, the hydroxyethylamino-

anthraquinones were not affected by cisplatin resistance mechanisms. The initial evaluation of the four chloroethylaminoanthraquinones in A2780CisR (2780CP) and CHCisR cell lines (Table 6. 3. and 6. 4.) showed that ZP282, ZP285 and ZP289 exhibited a weak cross-resistance to cisplatin, whilst ZP281 remained unaffected. These observations were confirmed when the compounds were re-investigated in a comparative study with A2780cisR (2780CP) (Table 6. 8). However, in this assay three of the mustard anthraquinones demonstrated 16 fold resistance, twice that of cisplatin. Although both assays established similar trends amongst the compounds the large RF value difference for identical cell lines may reflect the conditions under which cell growth occurred in the presence and following the removal of drug.

A2780CisR (2780CP) is a cisplatin resistant variant in which resistance is due in part to the lack of expression of the hMLH1 protein involved in mismatch repair (Drummond *et al.*, 1996) (section 1. 5. 1. 4). The effect of re-introducing the *hMLH1* gene into cell line B1 (Table 6. 8) was to restore partial sensitivity to nearly all drugs. From this it can be assumed that a lack of mismatch repair can affect chloroethylaminoanthraquinone cellular resistance, however it is likely that other resistance mechanisms also exist in this cell line. These may include enhanced DNA repair through the induction of the *ERCC1* mRNA expression (Li *et al.*, 1999) and elevated levels of glutathione (GSH) (Parker *et al.*, 1991).

The investigation of all compounds in cell line SKOV-3 which shows intrinsic resistance to cisplatin both *in vitro* and *in vivo* (Kelland *et al.*, 1992) demonstrated a good response. Resistance in SKOV-3 has been linked to a deficient tumour suppressor gene p53 which reduces the ability of a cell to undergo apoptosis (Ormerod *et al.*, 1996).

The cell line 2780AD overexpresses the drug efflux pump (Pgp), and is consistent with the MDR1 phenotype which confers resistance to natural products such as the anthraquinones (section 1. 5, 1. 1.). The compounds tested, with the exception of ZP281 were found to be extremely cross-resistant in 2780AD. ZP281 is unsymmetrical with an alkylating function confined to one side arm, this feature distinguishes it from the other drugs in terms of structure and may be an important factor in its poor substrate specificity

for Pgp. The circumvention of the MDR1 mechanism has been described previously for the morpholinoanthracyclines and was suggested to be attributed to their lipophilic nature as this promotes rapid drug uptake into cells (Johnston *et al.*, 1987; Coley *et al.*, 1993). A similar mechanism of drug uptake may exist for ZP281. Once the drug is bound covalently to DNA drug efflux from the cell nucleus would become ineffective.

The seven compounds were further evaluated at short term (4h) drug exposure to compare the effects of covalent and non-covalent binding on the rate of cell growth inhibition. Because nitrogen mustards such as mechlorethamine are non-cell cycle specific (section 1. 4) a more immediate cytotoxic effect was predicted for the mustard anthraquinones. All compounds showed enhanced potency after 24 hours in both resistant and non-resistant cell lines consistent with cell cycle progression. However, no difference was found in the inhibitory effects of the two classes of compound after short term exposure to cells, perhaps suggesting that the chloroethylaminoanthraquinones and hydroxyethylaminoanthraquinones have similar cell cycle specificities. The closeness of the IC₅₀ values obtained for the covalent and non-covalent binding drugs, particularly structurally similar ZP285 and ZP265 suggests that it is likely that some of the alkylating drug is lost to cellular nucleophiles before it goes on to bind with its target. Hence, the observed cytotoxicity of the chloroethylaminoanthraquinones may be an effect of a reduced concentration of drug.

The cytotoxicity of mitoxantrone is related to its ability to persistently trap the 'cleavable complex' by continually reforming with its nuclear target topo II (Smith *et al.*, 1992; Feofanov *et al.*, 1997). Although in this thesis the studies of drug-topo II inhibition were inconclusive (Chapter 5) they do not discount the fact that the seven compounds tested may target this enzyme. If this is indeed the case, the lack of topo II isoform selectivity demonstrated by ZP285 and ZP281 may be recognised as advantageous because cell growth inhibition would not be dependent on the topo II α status of the cell but would be effected by either isoenzyme. This is a positive response towards at-MDR where the loss of one topo II isoform has been linked to reduced drug sensitivity and resistance (Harker *et al.*, 1991; Mirski *et al.*, 1993). However in order to substantiate these suggestions it necessary to

investigate further the topo II inhibition of the seven novel anthraquinones (for discussion and future work, see Chapters 5 and 7).

In summary, all the compounds assayed demonstrated excellent cytotoxicity in all cell lines tested. The seven most potent drugs were closely compared for their activity in order to distinguish differences between covalent and non-covalent binding target interaction. This became apparent when only three chloroethylaminoanthraquinones showed cross-resistance with cisplatin in A2780CisR. ZP281 also a mustard remained active in all resistant cells tested at comparable concentrations to the parent wild type cells. The similarity in IC_{50} values obtained from both classes of drug infers that some chloroethylaminoanthraquinone is lost to cellular nucleophiles before it reaches its target and so the true effect of these drugs is not reflected in this study. To conclude, the drug ZP281 showed good activity in all ovarian cell lines which corresponded with lack of cross-resistance to cisplatin and doxorubicin. ZP281 is therefore an interesting candidate for further study as a drug that may possibly inhibit topo II and evade multi-drug resistance mechanisms.

CHAPTER 7 SUMMARY, FUTURE WORK AND CONCLUSION

The nitrogen mustards are bifunctional anti-tumour agents that are cytotoxic through an ability to alkylate DNA via the formation of covalent inter and intra-strand crosslinks. Their high reactivity and lack of specificity leads to much drug being lost to cellular nucleophiles before it reaches its target (see section 1. 4). Previous groups have identified that by linking the mustard function to a DNA affinic chromophore an increase in DNA specificity and cytotoxicity can be achieved. The DNA intercalating drug mitoxantrone inhibits topo II by reversible association. By combining an aliphatic nitrogen mustard function with an anthraquinone chromophore such as that found in mitoxantrone it is envisaged that such drugs would have improved target specificity and the potential to covalently bind the 'cleavable complex' and cause a permanent inhibition of the topo II enzyme.

In this study a number of hydroxyethylaminoanthraquinones were synthesised and reacted further to give a series of nitrogen mustard anthraquinones (chloroethylaminoanthraquinones) comprised of mono- or bis-alkylating chloroethylaminoalkylamino side chains substituted onto either the 1 position of the anthraquinone chromophore, or onto the 1, 4 position of either a 5,8-dihydroanthraquinone or 5,8-dihydroxyanthraquinone chromophore. The hydroxyethylaminoanthraquinones were isolated with relatively poor yield, between 20-50% depending on the purity of the side chains used. Conversion to the mustards was achieved by reacting with triphenylphosphine/carbon tetrachloride as the halogen donor to give products in approximately 80% yield. Further to this, the potential for enhancing the selectivity of the chloroethylaminoanthraquinones for hypoxic conditions was explored.

Bioreductive prodrugs are compounds that are non-toxic to normal cells but can be metabolically activated under reductive conditions to target hypoxic cancer cells (Workman, 1992). Hypoxic cells maintain viability despite existing at an oxygen

concentration that is suboptimal for cell growth and metabolism. They constitute only a minor subpopulation in mostly solid tumours but are found to be resistant to chemotherapy through poor drug accessibility (Workman *et al.*, 1993). AQ4N is an anthraquinone prodrug (Patterson, 1989) that is soon to go into clinical trials. The electrically neutral side chains of this compound diminish its affinity for DNA and consequently drug cytotoxicity. Under aerobic conditions AQ4N is stable, however in the absence of oxygen it is reduced enzymatically to the potent metabolite AQ4 (Patterson, 1993). By analogy it can be rationalised that a chloroethylaminoanthraquinone N-oxide would also have the potential be effective in the selective treatment of hypoxic tumours. Although previous work has suggested that the aliphatic bifunctional mustard moiety can be stabilised to hydrolysis by conversion to an N-oxide (Connors, 1988) the attempted synthesis of ZP293 N-oxide hydrochloride (section 2. 6.) resulted in the isolation of an unstable product. This proved to be consistent with previous reports of the synthesis of both aliphatic (Owari *et al.*, 1953) and aryl nitrogen mustard N-oxides (Tercel *et al.*, 1995) which were found to degrade to cyclic hydroxylamines. In order to prevent similar instabilities it is possible that the synthesis of future nitrogen mustard N-oxides may be achieved by designing substituted alkylating side chains that are constrained and so unable to cyclise to active metabolites. This approach is presently ongoing in this laboratory.

The novel anthraquinones were investigated to determine their affinity for DNA through intercalative binding. Spectroscopic titration of the hydroxyethylaminoanthraquinones gave DNA association constants (K_1) in the range $0.2\text{--}1.2 \times 10^7 \text{M}^{-1}$, consistent with mitoxantrone and its 5,8-dihydro derivative ametantrone (Kapuszcinski *et al.*, 1985; Lown *et al.*, 1985). In contrast, the chloroethylaminoanthraquinones did not show isosbestic behaviour and instead displayed a massive change in the visible region on addition to buffer and calf thymus DNA. In view of this, it is possible that high salt concentration, although discouraging surface binding, may encourage drug dimerisation. Previous reports have shown that the formation of the aziridinium ion is reversible in the presence of high chloride ion concentration (Chang *et al.*, 1979). Indeed it is conceivable that high salt concentration may be the cause of the

spectral shifts and lack of an isosbestic point for these hydrophobic mustard anthraquinones. O'Connor *et al.*, (1992) determined the DNA binding constants of aniline mustards linked to acridine under salt free conditions and so it is advisable that future work be conducted in the absence of sodium chloride.

DNA thermal denaturation studies identified that drug-helix stability increases with substitution onto the anthraquinone chromophore, and is influenced by side chain type and the chromophore hydroxyl groups. A similar relationship was established when comparing increasing cytotoxicities within the chloroethylamino- and hydroxyethylaminoanthraquinone class of drug (see Chapter 6). The alkylating anthraquinones were found to apparently bind weakly to DNA and only ZP281 stabilised the helix to some extent. This did not reflect the DNA crosslinking studies performed in Chapter 4 which demonstrated that a one hundred fold lower concentration of drug could successfully inhibit DNA strand separation. The small ΔT_m values of the mustard anthraquinones may arise from the fact that significant binding would be associated with DNA crosslinking and the resultant abolition of DNA melting characteristics. ZP281 is distinguished by having only one alkylating side chain and so would provide fewer DNA cross-linked sites and so allowing DNA melting to take place. This can be substantiated to some extent as ZP281 was found to be the weakest DNA strand separation inhibitor assayed (see Chapter 4).

The DNA alkylation sequence of four chloroethylaminoanthraquinones ZP281, ZP282, ZP285 and ZP289 produced an altered pattern of alkylation compared to that of the untargeted mustard mechlorethamine. This followed the principle that an intercalating chromophore dictates the site of alkylation. Furthermore, preferential selection for 5'PyG bases could be identified with sites of mitoxantrone chromophore intercalation (5'CG) (Bailly *et al.*, 1996) and potential sites of DNA inter-strand crosslink formation. Although it is recognised that untargeted nitrogen mustards such as mechlorethamine crosslink DNA at 5'GNC complementary base pairs (Osborne *et al.*, 1993) it is likely that an aliphatic nitrogen mustard moiety linked with a short side chain to an intercalated chromophore would restrict alkylation to nearby nucleophilic centres. The

chloroethylaminoanthraquinones could therefore only effectively crosslink the base pairs at the intercalation site. This may be confirmed by the use of molecular modelling which would establish if such a conformation was possible at 5'PyG DNA sequences. Bidirectional footprinting may also be useful to identify inter-strand crosslinked sites on the complementary strand of DNA (Hartley *et al.*, 1993). As 5'GNT inter-strand crosslinks have been identified for both mechlorethamine and L-phenylalanine mustards (Osborne *et al.*, 1995) it would also be interesting to determine if the chloroethylaminoanthraquinones are able to form crosslinks with thymine.

Three chloroethylaminoanthraquinones: ZP281, ZP285 and ZP289 demonstrated growth inhibition of yeast strains transfected with either human α or β , or wild type *Sc. ce* topo II isoforms. However the observed lack of drug isoform selectivity led to inconclusive evidence for topo II inhibition as a mechanism of cytotoxicity (see Chapter 5). In favour of topo II inhibition, the (5'PyG) sequences identified from chloroethylaminoanthraquinone-DNA alkylation studies (see Chapter 4) coincide with those previously identified with mitoxantrone topo II inhibition (5'C/T) and this supports the postulation that drugs of the same class stimulate identical topo II-DNA cleavage sites (Capranico *et al.*, 1993; Paoletti *et al.*, 1993). Moreover, because a high sequence homology exists between the α and β active sites, drug-covalent binding of nucleophilic sites (amino acids) within this region could potentially afford almost uniform levels of inhibition of both isoenzymes. Alternatively, extensive DNA-drug crosslinking (demonstrated at low concentrations in Chapter 4) may serve to distort the helix and prevent the enzyme from recognising DNA as a substrate. In this case the chloroethylaminoanthraquinone would act as 'DNA binding inhibitor' and not a 'topo II poison' (see Fig. 1. 2A and B).

In contrast, the hydroxyethylaminoanthraquinones (ZP257, ZP265 and ZP275) precipitated from the media at high drug concentration (100 μ M), and at low concentrations were found to be inactive possibly due to poor drug penetration of the yeast cell wall. The use of other permeability mutations to *ISE2* for example *MDS1* (Mixed Drug Selection 1) may benefit the outcome of future inhibition studies that utilise this assay (personal comm. Dr J. Jenkins).

A more accurate assessment of topo II selectivity can be made by studies with either purified topo II enzyme (α or β), or by topo II mediated kDNA decatenation (Smith *et al.*, 1997). These are assays designed to determine the specific targeting of topo II by gel electrophoresis. Alternatively, the DNA footprinting assay may perhaps be utilised to reveal drug-DNA-topo II adducted binding sites (Panoussis *et al.*, 1994). As lysine residues are highly nucleophilic and known to be important in DNA recognition by topo II (Li *et al.*, 1997), it may be possible to establish drug alkylation specificity of the topo II protein active site.

All the anthraquinone compounds assayed showed excellent activity in the ovarian carcinoma cell lines. Seven compounds ZP257, ZP265, ZP275 (hydroxyethyl-anthraquinones) ZP281, ZP282, ZP285 and ZP289 (chloroethylaminoanthraquinones) demonstrated comparable cytotoxicity with doxorubicin. In cisplatin resistant cell line A2780cisR (CP70), the hydroxyethylanthraquinones showed no cross-resistance confirming that they avoid the mechanisms of resistance usually associated with alkylating agents. In contrast, the chloroethylaminoanthraquinones with the exception of ZP281 (RF 2.9), were 16 fold resistant. Six compounds with the exception of ZP281 showed cross-resistance in the doxorubicin resistant cell line A2780AD. Furthermore, when the mismatch repair gene *hMLH1* was re-introduced into B1, cytotoxicity was partially restored to the chloroethylaminoanthraquinones suggesting that mismatch repair is a mechanism of resistance that can directly affect the sensitivity of these drugs.

The data derived from the cytotoxicity study of the novel-anthraquinones has uncovered that ZP281 may avoid the mechanisms of resistance associated with both anthraquinone and nitrogen mustard resistance. To further support this, it would be advantageous to determine the effects of ZP281 in cell lines displaying at-MDR such as the ovarian cell line ESR-2 which has demonstrated resistance to doxorubicin through reduced expression of topo II (Bielack *et al.*, 1995), or in cell lines that overexpress either topo II isoform (Houlbrook *et al.*, 1996). These assays may also serve to confirm drug selectivity for the enzyme.

Attempts at covalent drug-topo II inhibition have been made previously. Koyama *et al.*, (1988 and 1989) synthesised a series of aliphatic mustards linked to chrysophanol and emodin as potential irreversible inhibitors of the DNA-topo II complex. Although some of these compounds showed good enzyme inhibition and enhanced anti-leukemic properties they were not carried forward for further study because they were not significantly potent (Watanabe *et al.*, 1991). More recently, an azido derivative of the DNA intercalating agent ethidium (8-azidoethidium) has been developed to explore the effects of covalent binding on enzyme inhibition. Following chromophore intercalation into DNA the drug is photoactivated to an irreversible binding agent that can increase the number of single and double strand breaks created by topo II (Marx *et al.*, 1997). However this method of activation suggests that it is unsuitable to be used as a drug in cell systems.

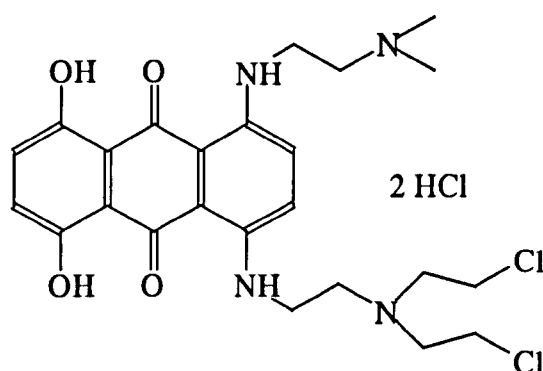


Fig 7. 1 Structure of compound ZP281

The work in this thesis has identified ZP281 (Fig. 7. 1) as a potential irreversible inhibitor of the DNA-topo II cleavable complex. ZP281 has demonstrated an ability to inhibit DNA strand separation at nanomolar concentrations and to alkylate DNA base pairs at 5'PyG sequences which are consistent with previously identified sites for topo II inhibition. The cytotoxicity data indicates that ZP281 is extremely potent with nanomolar IC₅₀ values in all cell lines tested. It is not cross resistant with doxorubicin and is therefore unaffected by the overexpression of P-glycoprotein, the drug efflux pump. Furthermore, its relative low cross-resistance in the cisplatin resistant cell line demonstrates that it is only

minimally affected by cisplatin resistance mechanisms and the mismatch DNA repair process. ZP281 differs from the other chloroethylaminoanthraquinones by comprising of two unsymmetrically substituted side chains. The alkylating function being confined to only one side arm. It is possible that this configuration is responsible for the evasion of these resistance mechanisms. ZP281 is therefore a good candidate for further study.

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APPENDIX 1 : STANDARD EXPERIMENTAL PROCEDURES

Chapter 3

A1. 1 PREPARATION OF DNA SOLUTION

Calf thymus DNA (200mg) was dissolved into a sterile flask containing low ionic strength buffer (100ml) (see appendix 2) to give a concentration of 2mg/ml. This process was repeated using high ionic strength buffer (see appendix 2). The two flasks were then covered to exclude light and the DNA left to stir at 4°C for approximately 5 days. After this time each solution was filtered through glass wool into a series of sterile containers. A sample was taken from each flask and diluted ($\times 50$) into dH₂O and its absorbance measured at 260nm. By taking into account that the molar extinction co-efficient of calf thymus DNA per nucleotide is 6600cm⁻¹ at 260nm, Beer-Lamberts Law was applied to determine the nucleotide concentration of the DNA.

Chapter 4

A1. 2 LINEARISATION OF PLASMID DNA pBR322

A combination of the following was added to a sterile eppendorf and vortex mixed: Plasmid DNA pBR322 (15µg /60µl), react 3 buffer (10µl), and dH₂O (27µl). To this reaction mixture was then added the restriction enzymes BamHI/ SalI (3µl) to make up a total volume of 100µl. The sample was then incubated at 37°C for 60 min. To precipitate the DNA, sodium acetate (3M, pH 3.3, 30µl) and 95% ethanol (300µL) were added to the eppendorf, the sample vortex mixed and frozen in a dry ice/ ethanol bath for 20 min. Following centrifugation at 15,000×g, 4°C for 15 min, the supernatant was discarded and the pellet washed with 70% ethanol. After drying the pellet by lyophilisation, the DNA was resuspended in dH₂O (33µl) and 1µl removed to verify its linearity by electrophoresis.

A1. 3. DNA ALKYLATION STUDIES

A1. 3. 1 DRUG TREATING THE DNA

The DNA plasmid pBR322 was linearised according to the method in appendix A1. 2. The following constituents were added to a sterile eppendorf: Linearised plasmid DNA pBR322 (0.5µg/10µl per PCR reaction), TEoA buffer (5µl) and drug solution (1µl) to give final assay drug concentrations of 100nM, 50nM, 10nM, 5nM and a mechlorethamine (control drug) concentration of 2.5µM. Each sample was made up to 50µl with dH₂O, mixed and then incubated for 1 h at 37°C. To precipitate the DNA, aqueous sodium acetate solution (3M, pH 3.3, 30µl), dH₂O (40µl) and ethanol (300µl) were added to the sample, which was vortex mixed and frozen on a dry ice/ethanol bath. Following centrifugation at 1300rpm, 4°C for 15 min, the supernatant was removed and the pellet washed twice with 75% ethanol (100µl) and dried by lyophilisation. The DNA was then resuspended in dH₂O (40µl /0.5µg per sample).

A1. 3. 2. END LABELLING THE PRIMER WITH ³²P-γATP

The following constituents were added to a sterile eppendorf and mixed: Primer (5.5µl), forward buffer 5× (5µl), ³²P-γATP soln (1µl) and the volume made up to 24µl with dH₂O. To this was then added the enzyme T4 kinase (1µl) and after careful mixing the sample was incubated at 37°C for 1 h. The resulting incubate (25µl) was then passed through a BIO-SPIN column at 1100rpm, 20°C for 4 min and the eluent collected. The BIO-SPIN column was then re-washed with dH₂O (25µl) and the eluents combined to give a total volume of 50µl.

A1. 4 DNA CROSSLINKING ASSAY

A1. 4. 1. DEPHOSPHORYLATION OF LINEARISED PLASMID pBR322

The following constituents were added to a sterile eppendorf and mixed: Linearised plasmid pBR322 DNA (79µl), BAP buffer 5× (20µl) and BAP (1µl). The sample was then incubated at 65°C for 60 min. After the sample had cooled to room temperature the DNA was extracted twice with phenol:chloroform:isoamyl alcohol (1:24:1) (100µl) and once with

chloroform:isoamyl alcohol (24:1) (100 μ l). The DNA precipitated and the pellet dried as described above and resuspended in dH₂O (17 μ l per 5 μ g of DNA).

A1. 4. 2 5' END LABELLING OF LINEARISED, DEPHOSPHORYLATED DNA

T4 polynucleotide kinase (1 μ l) was added to a sterile eppendorf containing a mixture of linearised dephosphorylated DNA pBR322 (5 μ g) in dH₂O (17 μ l), forward reaction buffer 5 \times (5 μ l) and γ ³²P-ATP soln (2 μ l) and the reaction mixture incubated at 37°C for 30 min. The resulting incubate (25 μ l) was then centrifuged at 1100rpm for 4 min through a pre-prepared BIO-SPIN column and the sample collected. The BIO-SPIN column was then further washed with dH₂O (25 μ l) by centrifugation for 4 min, and the resulting sample collected to give a total sample volume of 50 μ l. The DNA was precipitated by adding to the sample 7.5 M ammonium acetate (25 μ l) and 95% ethanol (150 μ l), and the pellet recovered and dried as above. To further remove any undissolved protein, the pellet was resuspended in dH₂O (50 μ l), precipitated in 95% ethanol (100 μ l), centrifuged, dried and re-suspended dH₂O (20 μ l).

APPENDIX 2: BUFFER SOLUTIONS AND MEDIA

Chapter 2

HPLC Mobile phase

(a) Methanol (999cm³)

Trifluoroacetic acid (1.0 cm³)

(b) dH₂O (999cm³)

Trifluoroacetic acid (1.0 cm³)

Chapter 3

High ionic strength buffer pH 7.2 (0.5M NaCl)

Sodium chloride (29.22g)

Tris-Base (0.96g)

dH₂O (1dm³)

pH 7.2 adjusted with HCl

Low ionic strength buffer pH 7.2 (0.05M NaCl)

Sodium Chloride (2.92g)

Tris-Base (0.96g)

dH₂O (1dm³)

pH 7.2 adjusted with HCl

Chapter 4

TEA (10×) buffer

Tris-base (121.1g)

EDTA (3.72g)

Acetic acid (58g)
dH₂O (1dm³)
pH 8.1 adjusted with HCl

Strand separation buffer

DMSO (30g)
EDTA (0.0192g)
bromophenol blue (0.04g)
xylene cyanol (0.04g)
dH₂O to make up a final volume of (0.1dm³)

TEoA buffer

Triethanolamine (3.75g)
EDTA (0.192g)
dH₂O (1dm³)
pH 7.2 adjusted with HCl

Formamide dye

Bromophenol blue (3mg)
Cyanol (3mg)
0.5M EDTA (0.4ml)
Formamide (9.6ml)

Chapter 5

Yeast synthetic media

Yeast nitrogen base (6.0g)
Glucose (40.0g)
NaOH (0.01g)

Amino acid mixture (-LEU)(0.70g)
dH₂O (1dm³)

A2. 1 PREPARATION OF THE ELECTROPHORESIS RUNNING GEL

TBE Buffer (10×) (stock solution)

Tris base (121.1g)
Boric Acid (51.35g)
EDTA (3.72g)
dH₂O (1dm³)
pH 8.0 adjusted with HCl

The following solutions were prepared:

a) Polyacrylamide gel:

Sequagel 6 (80.0g)
Complete mix (20.0g)
TBE buffer diluted into dH₂O 1 in 10 (0.1dm³)

b) 10% APS:

APS (1.0g)
dH₂O (0.01dm³)

c) Gel casting solution:

TEMED (70μl)
10% APS (50μl).
TBE buffer (1/10) (0.01dm³)

The polyacrylamide gel (0.01dm³), TEMED (40μl), 10% APS (340μl), TBE buffer (diluted 1 in 10 into dH₂O) (90ml) were mixed together to form a thin film coating in between two vertical glass plates (1m×0.4cm) which were held in place together with plastic holders and a gel caster (c).

APPENDIX 3 : HPLC ANALYSIS

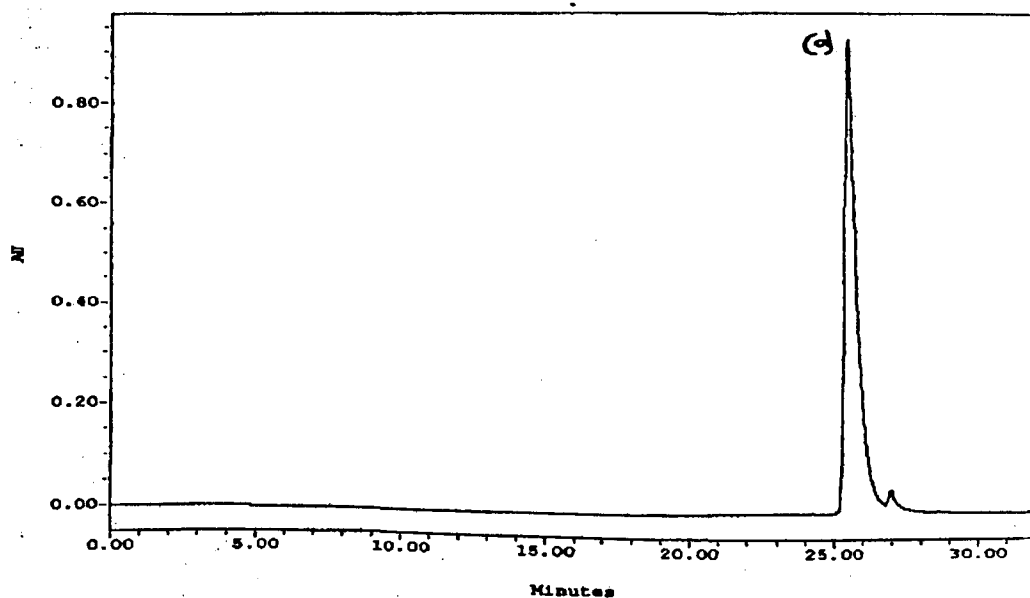


Fig. A3.1 The HPLC profile of ZP1

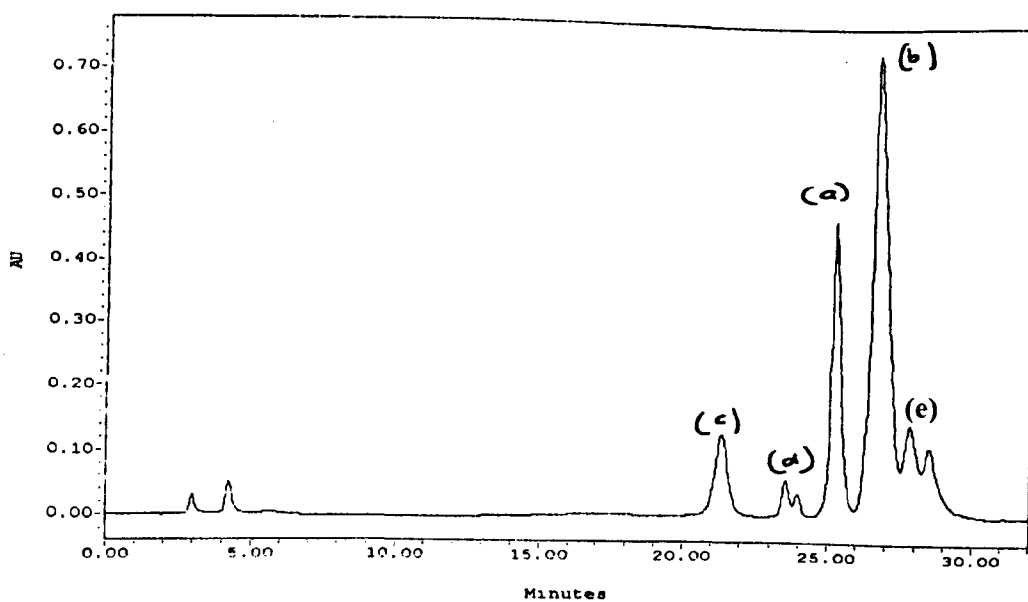


Fig. A3.2 The HPLC profile of the reaction of ZP1 with thionyl chloride after 24h at room temperature. Peak a) Starting compound ZP1. b) Required product ZP293; c), d), and e) Unknown impurities.